IL-12 Initiates Tumor Rejection via LTi Cells Bearing the Natural Cytotoxicity Receptor NKp46

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IL-12 INITIATES TUMOR REJECTION VIA LTi CELLS BEARING THE NATURAL CYTOTOXICITY RECEPTOR NKp46

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Disclaimer

The thesis is based on the following publication:

- ***IL-12 initiates tumor rejection via lymphoid tissue-inducer cells bearing the natural cytotoxicity receptor NKp46***

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Abbreviations

Ab	Antibody
Ag	Antigen
AICD	Activation-induced cell death
αGalCer	Alpha-galactosylceramide
APC	Antigen-presenting cell
bp	Base pair
BM	Bone marrow
CRAd	Conditionally replicating adenovirus
cNK	conventional Natural killer
CP	Crypto Patches
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
FGF	Fibroblast growth factor
γδT	gamma delta T cell
HEV	High endothelial venules
Ig	Immunoglobulin
IFNγ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitic oxide synthase
i.p.	Intra peritoneal
i.t.	Intra tumoral
i.v.	Intra venous
kDa	KiloDalton
KDC	killer Dendritic cell
KIR	Killer inhibitory receptors
LN	Lymph node
LTα	Lymphotoxin alpha
LTi	Lymphoid Tissue inducer
mAb	monoclonal Antibody
MCA	3-methylcholanthrene
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MLR	Mixed lymphocyte reaction
NALT	Nasopharyngeal-associated lymphoid tissue
NCR	natural cytotoxicity receptor
NK	Natural killer cells
NKT	Natural killer T cells
PP	Peyers Patches
RAG	Recombination-activation gene
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
s.c.	Subcutaneous
SLT	Secondary lymphoid tissue
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGFβ	Transforming growth factor beta
T_H	T helper cells
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
WT	Wild-type
LTβR	Lymphotoxin beta receptor

Aim of the Thesis

It was the main goal to understand the cellular and molecular underpinnings relevant for tumor protection and rejection. Whilst the potency of IL-12 as a tumor-suppressing cytokine has been clearly established, the fact that systemic application led to severe adverse effects has halted the development of IL-12 threapies in humans. However, to this day, the molecular and cellular events are ill-understood. By defining the mechanistic underpinnings, we wanted to shed light onto how tumor cells and immune cells interact in general and in particular will enhance the therapeutic targeting of malignancies in humans.

Summary

Lymphocytes and their secreted cytokines play a critical role in tumor control and elimination. In a variety of tumor models, Interleukin-12 has been shown to repress tumor growth. The tumoricidal activity of IL-12 is widely held to be mediated by the activation and polarization of NK and type 1 T helper cells respectively. The purpose of this project was to specifically determine the precise mechanism by which IL-12 mediates tumor suppression rather than investigating its potential clinical application. By systematic analysis of the immune response to an Interleukin-12 secreting melanoma cell line (B16-F10) we found that tumor suppression is mediated independently of T lymphocytes or NK cells. We discovered that IL-12 initiates powerful local anti-tumor immunity by stimulating a subset of lymphoid tissue inducer cells dependent on the transcription factor RORγt. This report not only attributes a clear novel function to this only recently identified cell type in tumor immunology but also raises the possibility of therapeutically stimulating RORγt-dependent lymphoid tissue inducer cells in tumors as a new strategy for the eradication of solid tumors in patients.

Zusammenfassung

Lymphozyten und deren sekretierten Zytokine spielen eine wichtige Rolle bei der Kontrolle und Eliminierung von Tumoren. In vielen verschiedenen Tumor-Modellen wurde gezeigt, dass Interleukin-12 die Fähigkeit besitzt, das Tumorwachstum zu unterdrücken. Diese anti-tumor Aktivität von Interleukin-12 wird weitgehend der Aktivierung und Polarisierung von NK Zellen und Typ 1 Helfer Zellen zugeschrieben. Das Ziel dieses Projekts war in erster Linie das Analysieren des präzisen IL-12-vermittelten Mechanismus welcher zur Unterdrückung des Tumorwachstums führt und nicht das Erforschen einer potentiellen klinischen Applizierung für eine neue Therapie. Als Model verwendeten wir eine Melanoma-Zelllinie (B16-F10) die stetig Interleukin-12 sekretiert. Durch systematisches analysieren der Immunantwort nach Injektion dieser Zelllinie in die Maus haben wir herausgefunden, dass die daraus folgenden Tumor-Unterdrückung unabhängig von T Lymphozyten oder NK Zellen erfolgt. Stattdessen konnten wir zeigen, dass IL-12 eine starke lokale anti-tumor Immunantwort hervorruft indem ein spezifischer Zelltyp der zur Klasse der Lymphgewebe-Induzierer Zellen gehört stimuliert wird. Dieser Zelltyp ist abhängigen von dem Transkriptionsfaktor RORyt. Unsere Arbeit beschreibt nicht nur eine klare neue Funktion für diesen erst kürzlich entdeckten Zelltyp im Rahmen der Tumorimmunologie, sondern erbringt auch neue Möglichkeiten für therapeutische Maßnahmen durch die Stimulierung von RORyt-abhängigen Lymphgewebe-Induzierer Zellen im Tumor als neue Strategie für die Vernichtung von festen Tumoren in Patienten.

Introduction

Since the death toll from infectious diseases has declined in the western world, cancer has become second-ranking cause of death right after heart diseases. It affects people at all ages with the risk for most types increasing with age. After a quarter century of research, cancer has been revealed as a disease involving dynamic changes in the genome, whereby normal cells from a distinct tissue start to uncontrollable proliferate. Cancer refers to a malignant tumor. In comparison to benign tumors, they harmfully grow in healthy tissues and eventually metastasize into other tissues causing damage of vital structures and functions throughout the human body.

The Properties of Cancer

Healthy normal cells have distinct regulatory circuits that govern normal cell proliferation and homeostasis. Cancer cells however, clearly show defects in these regulations. The research over the past decades has revealed a small number of molecular, biochemical and cellular traits, shared by most and perhaps all types of cancer. There are specific events that govern the transformation of normal cells into malignant cancer, which are widely known as the 'hallmarks of cancer' (**Image 1**). Hanahan and Weinberg [1] suggested that the immense recording of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth.

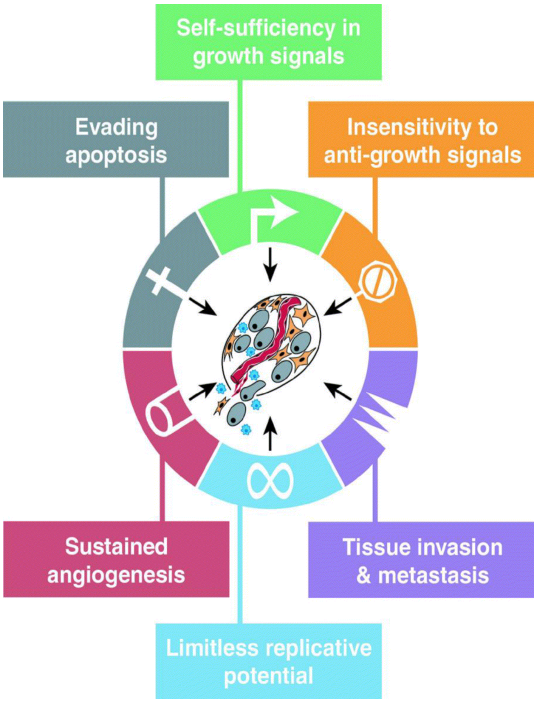

Image 1 Hallmarks of cancer [1]

Self-Sufficiency in Growth Signals

Signals in a cell's surrounding, such as diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecule, ensure a cell's movement from a quiescent state into an active proliferative state undergoing mitosis. Without these mitogenic growth signals, no normal cell will itself divide. This dependency of the cell on its surrounding tissue microenvironment ensures an important homeostasis in normal functioning tissues. A tumor cell however, can generate many of its own growth signals, which act in an autocrine fashion thereby creating a positive-feedback signaling loop. Most of these factors not only provide growth-stimulatory signals but also sabotage the immune response simultaneously. Interleukin-4 (IL-4) and IL-10 for instance have been described as growth factors for thyroid carcinoma [2]. But these two interleukins also promote the polarization of the immune response away from a favorable T helper (T_H) 1 tumor fighting response towards a T_H2 response. Another known growth signal involved in the pathogenesis of prostate cancer, renal cancer and myeloma is IL-6. IL-6 signaling is involved in STAT3 activation thus will lead to inhibition of inflammatory responses and crosstalk between innate and adaptive immune responses [3], which are needed in a successful anti-tumor combat. Furthermore, receptor over-expression for growth signals can lead to hyper responsiveness to normal levels of growth signals in the surrounding [4].

Insensitivity to Growth-Inhibitory Signals

To further maintain a state of homeostasis, multiple anti-proliferative signals can maintain a cellular's quiescence. Like the growth signals, they act through transmembrane cell surface receptors coupled to intracellular signaling circuits and force the cell into a state of quiescent or to permanently abandon their proliferative potential. Cancer cell must overcome these anti-proliferative signals mostly by disrupting the blockade for progression from G1 into S phase [5]. Many tumors also disable components of the transforming growth factor beta (TGFß)-mediated signaling pathway by mutation or loss of its receptor and by mutation of the transcription factor SMAD4. Effector functions such as stimulation of regulatory T cell (Treg) proliferation, reduction of T cell proliferation, suppression of natural killer (NK) cell cytotoxicity, reduction of antigen (Ag) presentation by dendritic cells (DCs) and inhibition of interferon gamma (IFNγ), make TGFß to one of the most important anti-proliferative signals which may influence the immune system at different levels [6 7 8].

Evasion of programmed cell Death (Apoptosis)

The extracellular and intracellular environment inspects conditions of normality or abnormality of the cell and influences whether a cell should live or die. Cell surface receptors bind either survival or death factors [9] [10] and intracellular sensors detect abnormalities as DNA damage or survival factor insufficiency [11]. Two main pathways can induce the apoptotic program, the mitochondrial pathway and the death-receptor pathway. Tumor cells however acquired resistance toward pro-apoptotic signals by means of over expressing endogenous inhibitors of the mitochondrial pathway like BCL-2 or MUC1. Further, they may lose the functional expression of factors like APAF1 that are required to induce apoptosis [12].

Limitless Replicative Potential

Cells carry an intrinsic cell-autonomous program that limits their multiplication and acts independent of environmental and cell-to-cell signaling. Each cell possesses at the ends of chromosomes so called telomeres, which are composed of several thousand repeats of a short 6 base pair (bp) sequence element. Each replication during a cell cycle results in a loss of 50 to 100 bp of telomeric DNA from the ends of every chromosome. This progressive erosion of telomeres eventually causes unprotected chromosomal ends leading in death of the affected cell [13]. A tumor cell can overcome this senescence and acquired the ability to multiply without limit [14] [15]. Mostly they up regulate the expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeric DNA [16]. In addition, the replicative potential can be ensured by mutations in senescence-inducing proteins such as p53 [12].

Sustained Angiogenesis

The vasculature supplies the cell with oxygen and nutrients. While this supply during organogenesis is ensured by coordinated growth of vessels and parenchyma, angiogenesis is carefully regulated in the accomplished tissue. Tumors induce and sustain angiogenesis in order to progress to a larger size [17] [18]. They do so by over expression of vascular endothelial growth factor (VEGF) and / or fibroblast growth factor 1 (FGF1) and FGF2. All of them are crucial to overcome the state of vascular quiescence by mediating the ‘angiogenic switch’ [12].

Tissue Invasion and Metastasis

Cancers gain during their development the capability to generate cells that leave the primary tumor mass and eventually invade distant organs within the body with at least initially fresh nutrients and space. These metastases are the cause for 90 percent of human cancer deaths [19] and depend on all of the five above mentioned hallmark capabilities.

Cancer against the Immune System

From Cancer Immunosurveillance to Cancer Immunoediting

In the early 1900s, Paul Ehrlich first conceptualized a theory suggesting, that cancer cells frequently arise in the body but are recognized from the immune system as foreign and are therefore eliminated [20]. Back at that time, this hypothesis however could not be experimentally tested because so little was known about the molecular and cellular basis of immunity. Some 50 years later, this concept has been formally introduced as 'cancer immunosurveillance' by Thomas and Burnet [21] [22]. They suggested that a cell-mediated branch of the immune system has evolved to constantly inspect the host's tissues for invading pathogens and aberrant cells. Along with this theory, there had to be distinctive structures on tumor cells recognizable by the immune system. Many experiments followed aiming to test whether hosts with impaired immune systems would show evidence for increased incidences of spontaneous or chemically induced tumors (reviewed in Dunn *et al.* 2002 [23]). However, these studies were inconclusive and failed to prove or disprove the immunosurveillance hypothesis. New technologic advances in mouse genetics and monoclonal antibody production allowed new studies in the 1990s revitalizing and ultimately validating the cancer immunosurveillance concept (reviewed in Dunn *et al.* 2004 [24]).

It is likely that immunity generally prevents the formation of cancer in healthy individuals, but cancers clearly develop spontaneously in spite of this surveillance. The interaction between the immune system and tumors is obviously more complex than initially conceived. There has been growing recognition that immunosurveillance represents only one dimension of the complex relationship between the immune system and cancer [23] [25]. Whereas the immune system on one hand eliminates cancer cells, it also sculpts the immunogenic phenotype of tumors that eventually form in immunocompetent hosts and evade immune control. It has been shown, that the immune system also functions to promote or select tumor variants with reduced immunogenicity, thereby providing developing tumors with a mechanism to escape immunologic detection and elimination. These dual opposing functions of immunity are named "cancer immunoediting", a process consisting of three phases: elimination (also known as immunosurveillance or protection), equilibrium (persistence) and escape (progression) (**Image 2**) [26] [25] [24] [27] [12].

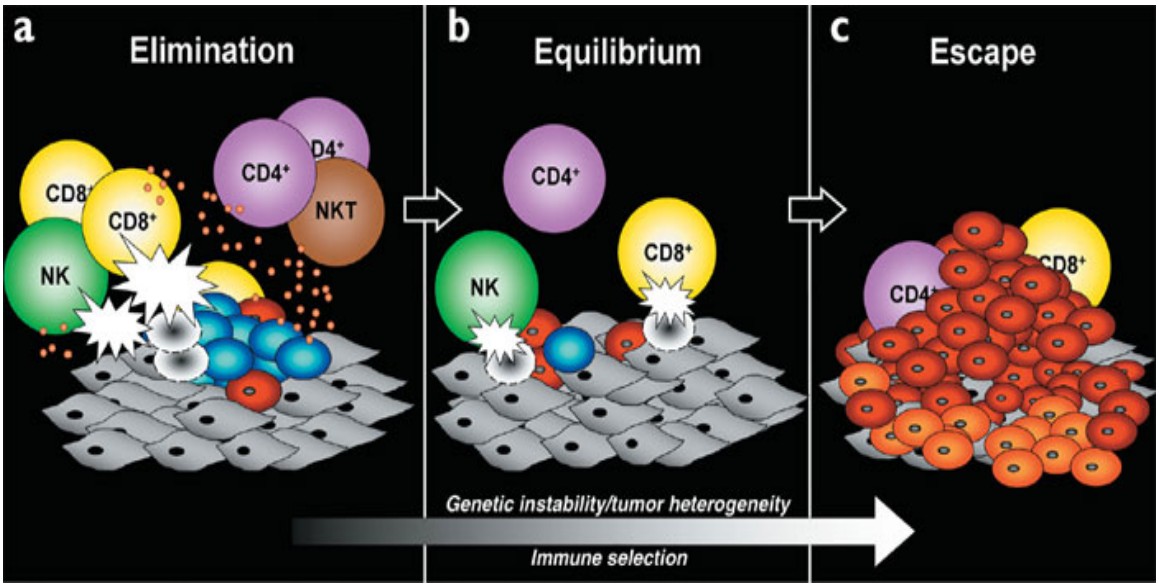

Image 2 The three E's of cancer immunosurveillance [23]

Cancer immunoediting covers three processes. (**a**) Immunosurveillance actually corresponds strictly speaking only to the phase of elimination, where the immune system actively fights the cancer. (**b**) During the equilibrium phase, eventually the immune system gradually selects and/or promotes the generation of tumor cell variants with increasing capabilities to survive immune responses. (**c**) Ultimately, these variants might escape the immune attacks and expand in an uncontrolled manner. Grey = non-transformed cells / blue = developing tumor cells / red = tumor cell variants / orange = additional tumor variants resulting of the equilibrium phase / small orange dots = cytotoxic activity of lymphocytes against tumor cells / green = NK cells / brown = NKT cells / yellow = $CD8^+$ T cells / purple = $CD4^+$ T cells.

Elimination

Elimination represents the classical phase of immunosurveillance. Until now, a great amount of research has clearly proofed the concept that deficiencies in key immunologic molecules enhanced the host susceptibility to both chemically induced and spontaneous tumors [26 28 29 30 31 32 33 34]. But what is the mechanism behind the extrinsic tumor suppressor to protect the immunocompetent host from the development of neoplasia? Numerous studies demonstrated that lymphocytes of both the adaptive and innate immune compartments prevent tumor development (reviewed in Loose and Van de Wiele 2009 [35]). Recently, there has also been evidence for the involvement of myeloid cells such as dendritic cells (DC) [36] and macrophages [37 38 39 40].

It is a common concept of the antitumor immune response, that the innate part of the immune system becomes alert to the presence of a growing tumor probably through stroma remodeling and the resulting tissue disruption which might produce pro-inflammatory molecules [24]. This molecules and probably molecules produced by the tumor cells themselves recruit a first line of defense to the tumor mass including NK cells, Natural killer T (NKT) cells, gamma delta T (γδT) cells and macrophages.

Natural killer cells

NK cells belong to the innate lymphocytes and can in contrast to T cells without Ag sensitization or clonal expansion, lyse NK-sensitive tumor targets[41]. Their production of cytokines may also modulate DC and cytotoxic T lymphocyte (CTL) maturation. NK cells simultaneously express an array of receptors that are categorized as either activating or inhibitory. Inhibitory receptors recognize major histocompatibility complex (MHC) class I molecules and other cell-surface molecules, essentially setting the threshold for NK cell activation. Important inhibitory NK cell receptors are members of the KIR family in humans and the C-type lectin-like Ly49 receptors in mice. Several of the activating receptors recognize MHC class I-like molecules that are encoded by pathogens and expressed on pathogen-infected cells or that are expressed only in the context of infection, DNA damage or stress. Natural cytotoxicity receptor (NCRs), including NKp46, are the major activating receptors [42 43 44 45]. Anti-tumor activities of NK cells include direct cytolysis in a perforin/granzyme-dependent manner. Upon interaction with the target cell, granules containing perforin and granzyme release their content. While perforin creates pores into the plasma membrane of the target cells, granzyme enters the cell and cleaves precursors of caspases leading to apoptosis of the target cell. Further, NK cells can activate other immune cells through secretion of IFNγ and other cytokines. It has been shown that depletion of NK cells following anti-asialo-GM1 treatment results in the mouse being more prone to developing chemical carcinogen 3-methylcholanthrene (MCA) -induced tumors than their wild-type counterparts [46].

Natural Killer T cells

NKT cells belong to the T cell pool but have additionally some unique features. They not only express a fully functional αß T cell receptor (TCR), though with a highly biased TCR repertoire, but also NK cell receptors. They are quite unusual in their MHC restriction as well, since they depend on a non-classical MHC molecule, CD1d, which preferentially presents glycolipids [47]. This CD1d-restricted NKT cell population has been divided into two subsets – type I NKT cells and type II NKT cells. Type I NKT cells have been characterized by a unique TCR Vα14Jα18 chain in the mouse and a Vα24Jα18 chain in the human along with a small number of possible TCR Vß chains. They recognize the glycolipid α-galactosylceramide (αGalCer).Type II NKT cells on the other hand, have a diverse TCR and do not respond to αGalCer, but still recognize lipids presented by CD1d [48]. There is yet another population of cells classified as NKT-like cells. Unlike the other two, they are restricted to classical MHC class I and II molecules but not the CD1 molecule and are therefore also termed CD1d-independent NKT cells [47], but will be here no further discussed. Upon stimulation, CD1-dependent NKT cells respond with a vast production of cytokines including IL-4, IL-10, IL-13 as well as IFNγ and Tumor necrosis factor alpha (TNFα) [49]. Depending on the activation, NKT cells can polarize the immune response in a T_H1 or T_H2 direction. Regarding the tumor immune response, activated NKT cells have been shown to mediate direct cytotoxicity in a perforin-dependent manner or stimulate NK cell and CTL antitumor cytotoxicity by the production of IFNγ. Indeed, *Jα281*$^{-/-}$ mice lacking a large population of Vα14Jα281- expressing invariant NKT cells, were found to develop MCA-induced sarcomas at a higher incidence than their wild-type counterparts [50]. On the other hand it has been shown, that NKT cells polarize the anti-tumor immunity in a less favorable T_H2 response. While type I NKT cells have been more often associated with tumor suppression, type II NKT cells have been shown to be involved in surveillance suppression [51] [47].

Gamma delta T cells

While most T cells express the TCRαß, a minority of 1-10 percent bears the TCRγδ, which are predominantly $CD4^-CD8^-$. Most of these cells are found in the intraepithelial lymphocyte (IEL) compartments of the skin, intestine and genitourinary tract. Even though the γ and δ TCR loci just like the α and ß ones may generate a vast degree of TCR diversity, only very little diversity is found in this type of T cells. Furthermore, these cells have Ag recognition properties fundamentally different from those of αß TCRs. They are not constrained by the selectivity and restriction of the MHC and therefore do not require peptide recognition and priming. The γδTCR structure is more similar to immunoglobulins (Ig) than it is to the αßTCR and has the potential to recognize a wide variety of Ags. Like NK cells they broadly recognize and immediately respond to cells that are missing self-recognition or are stressed/transformed. Via perforin/granzyme, they eventually perform a direct killing of the transformed cells or the early IFNγ production may stimulate NK cell and CTL antitumor cytotoxicity [52] [53]. Studies in *TCRδ*$^{-/-}$ mice, showed an increase susceptibility to tumor formation and a higher incidence of papillomas-to-carcinoma progression compared to wild-type mice [54].

Macrophages

Another player of the innate immune system are macrophages. They are capable of ingesting and digesting exogenous Ags, such as whole microorganisms, insoluble particles and endogenous matter, such as injured or dead host cells and cellular debris. The uptake of particulate Ag is an initial stimulus to activate the cell. However, the activity is often enhanced by secreted cytokines within their microenvironment, especially IFNγ. Apart from their cytotoxic activity through reactive oxygen intermediates (ROIs), reactive nitrogen intermediates and lysozymes, they are also potent Ag-presenting cells (APCs) and are able to secrete various cytokines, such as IL-1, TNFα an IL-6 which mostly promote inflammatory responses. Recently, there has been evidence, that micro environmental cytokines may polarize macrophages toward a so-called M1 or M2 phenotype, whereby each expresses specialized functional properties. IFNγ and microbial molecules as LPS induce activation of M1 macrophages, which can support a T_H1 immune response, microbicidal activity, immuno-stimulatory functions and tumor cytotoxicity. IL-4, IL-10, IL-13 and colony stimulating factor (CSF-1) in association with either IL-1R or TLR ligands on the other hand induce M2 macrophage activation and promote a T_H2 response, scavenging ability, promote tissue repair, angiogenesis, tissue remodeling and repair, which ultimately supports tumor progression [55].

Monocytes circulating in the blood will eventually further differentiate and lead to tissue specific macrophages, in the case of tumors they are called tumor-associated macrophages (TAM). Macrophages are often the most abundant immune cell population in the tumor microenvironment. These TAMs within tumor microenvironment tend to polarize towards a M2 phenotype supporting the tumor growth by the selection of a less favorable T_H2 anti-tumor response and the induction of angiogenesis [56].

Dendritic cells

DCs belong to the innate immune system and represent a heterogeneous population of cells defined by their anatomic distribution, phenotype, mode of Ag presentation and cytokine production profile. They not only instruct T and B cells, but also activate NK cells and produce interferons, thus linking the innate and adaptive immune system. Recently, there has been evidence for a novel DC subset which shows some unique phenotypic and functional properties [36]. Expression of markers such as CD11c and MHCII clearly relates to conventional DCs. However, they also express markers of the NK cell lineage such as B220 and NK1.1 and also inhibitory (Ly49 family members, NKG2A and KLRG1) as well as activating (NKG2D) receptors. Apparently this novel subset of DCs has not only Ag presenting skills but can also exert direct cytotoxic activity against cancer cells in vitro and in vivo and have therefore been termed killer DC (KDC) [57 58 59 60 61]. There have been different killer-mechanisms associated with KDC including perforin/granzyme-mediated [57] as well as TRAIL-dependent killing [62]. KDCs may not only participate in the effector phase of the immune response eventually leading to tumor elimination but also promote the release and immediate availability of tumor Ags in order to present them to T cells.

Beside the direct killing of a proportion of the tumor by the innate immune cells, a common outcome of the innate immune system encountering the tumor mass is the production of IFNγ which leads to the local production of chemokines that in turn recruit more cells of the innate immune system. IL-12, here most likely produced by tumor-infiltrating macrophages stimulates again the IFNγ production by these recruited innate cells, leading to a positive feedback loop boosting the immune response. Furthermore, this enhanced IFNγ production may already kill a certain amount of the tumor through different IFNγ-dependent processes like anti-proliferative, pro-apoptotic and anti-angiostatic effects [24]. There are several studies with support the notion, that endogenously produced IFNγ protects the host against the growth of transplanted tumors and spontaneous tumors [29 28 33 34 26].

Since tumor cells arise from the body's own cells, there clearly must exist tumor-derived immunogenic molecules that are qualitatively different form normal self-molecules in order for the immune system to elicit an effective immune surveillance. This combat of tumor cells may rely on tumor-specific Ags or other tumor Ags, like products of oncogenes or mutated tumor suppressor genes which can be shared by different cancer cells that potentially stimulate immune responses [63 64]. These tumor Ags are probably liberated by the effect of the innate immunity. DCs now may acquire these tumor Ags and migrate to the draining lymph node (LN), where they induce the activation of naïve tumor-specific adaptive immunity [24]. Until now, there have been lots of immunogenic human tumor Ags identified and segregated into the following classes: Differentiation Ags (e.g. melanocytes differentiation Ag, Melan-A/MART-1, tyrosinase, gp-100), Mutational Ags (e.g. abnormal form of p53), Over expressed / Amplified Ags (e.g. HER-2/neu), Viral Ags (e.g. EBV and HPV) and Cancer-Testis (CT) Ags (e.g. MAGE, BAGE, GAGE) [65 66]. But transformed cells may over express other molecular signpost through which they can be recognized by the adaptive as well as the innate immune system. One important molecule that has come up in several studies is the NKGD-activating receptor expressed on NK cells, γδT cells as well as CTLs. Different ligands of this receptor have been described to be expressed by a variety of tumors in humans and mice [25]

T lymphocytes

T cells belong to the cellular branch of the adaptive immune system. They arise in the bone marrow and migrate for maturation to the thymus. These naïve T cells express a unique cell surface antigen-binding molecule called the T cell receptor (TCR). Once encountering their antigenic peptide in context of MHC presentation, the presence of co-stimulatory signals (such as CD28-B7 interactions) and an additional set of cytokine signaling, they become activated. Class I and Class II MHC molecules interact with different co-receptors, CD8 and CD4 respectively, which allows the identification of $CD4^+$ T_H cells and $CD8^+$ CTLs. $CD8^+$ T cells, are referred to as CTLs given their ability to kill target cells or in this case tumor cells. They are capable to detect quantitative and qualitative antigenic differences in transformed cells. Differentiation of the CTLs leads then to the formation of modified lysosomes stuffed with perforin and granzyme and furthermore to an increased expression of death-activator-designated Fas-ligand (Fas-L) [67 68]. Upon interaction with the target cell, the lysosomes perform exocytosis and releases perforin and granzyme leading to perforation of the target cell and apoptosis.

More often however, apoptosis of the tumor cells is induced by the interaction of Fas-L with Fas. [69]. The binding triggers apoptosis through the classical caspase cascade [70]. Further, CTLs may influence target cells by the secretion of cytokines as TNFα or INFγ. TNFα engages its receptor on the target cell and triggers the caspase cascade, leading again to target-cell apoptosis. IFNγ induces transcriptional activation of the MHC class I Ag presentation pathway and Fas in target cells, leading to enhanced presentation of endogenous peptides by MHC class I and increases Fas-mediated target-cell lysis. $CD4^+$ T cells can be divided into T_H1 and T_H2 cells. The balance between these subsets has been shown to be critical in various immune responses, including antitumor immune responses. T_H1 cells produce mainly IFNγ. They are essential for the induction of cellular immunity. T_H2 cells on the other hand produce IL-4, IL-5 and IL-10 and play a key role in humoral immunity counteracting the T_H1-cell response. In terms of tumor immunity, Nishimura *et al.* showed that T_H1 cells induced a strong lymphocyte infiltration into the tumor mass and eradicated the tumor via cellular immunity. T_H2 cells on the other hand seemed to induce inflammatory responses at the tumor site and induced tumor necrosis [71]. Even though $CD4^+$ T cells have been shown to be sufficient by themselves in eliminating tumor cells in the absence of CTLs, more often however tumor-cell elimination requires both types of T cells [72 73 74 75]. Studies with mice lacking the recombinase activating gene (RAG) and therefore can not produce T cells, B cells nor NKT cells, showed upon MCA-induced sarcoma faster and greater frequencies of tumor development [26].

Taken together, these data not only highlight roles for both innate and adaptive immune components in the elimination phase of cancer immunoediting but also underline the complexity of the host's immune response to developing tumors.

Equilibrium

Equilibrium is the period of immune-mediated latency after incomplete tumor destruction in the elimination phase. This period in the course of the immune system / tumor interaction probably occurs prior to the detection of clinically apparent tumors. It is perhaps the longest of the three phases and may occur over many years in humans [76].

Initially, the immune system constrains the growth of tumors. But basically, a productive antitumor immune response can serve as a biological selective pressure that promotes the emergence of more aggressive tumor variants. Heterogeneity and genetic instability of cancer cells is possibly the principal force to generate new populations of tumor cells carrying mutations, which in time might lead to reduced immunogenicity. Ultimately, this dynamic interaction between immunity and cancer might eventually lead to tumor cells with resistance to the host's immunological blockade and therefore to escape. The enormous plasticity of the cancer cell genome is thought to arise from several types of genetic instability, including nucleotide-excision repair instability, microsatellite instability, and chromosomal instability [77], the latter of which may induce gains or losses of whole chromosomes.

Escape

Escape refers to the final outgrowth of tumors that have overcome immunological restraints of the equilibrium phase. This failing in consequent detection and removal of malignant cells is the result of various escape strategies established by tumor cells and basically refers to the 7th hallmark of cancer. Since adaptive as well as innate components of the immune system play its part in cancer immunosurveillance, tumors most likely would have to circumvent either one or both arm of immunity in order to achieve progressive growth. There are several possible mechanisms by which tumor cells can escape. Thus, it is likely that several of this tumor sculpting events must occur before the final immunogenic phenotype of malignant cell is ultimately established (reviewed in [35 78 79]).

Alteration in MHC class I antigen

MHC class I Ags are essential for the presentation of peptides such as tumor-associated Ags to CTLs. It has been shown, most if not all types of solid tumors show loss or down regulation of all or certain MHC class I allospecificities on cells [80 81 82 83]. This lesion in Ag processing and presentation pathways facilitates evasion from the adaptive immune recognition. The NK cell however shows enhanced activity under the condition of decreased MHC class I expression, since inhibitory signals through killer inhibitory receptors (KIRs) are diminished. This might compensate for the poor CTL response. But it has also been shown, that some leukemia cell lines up-regulate MHC class I expression, which delivers stronger inhibitory signals to NK cells [42].

Antigen loss

Tumor Ags, as any other antigenic protein, requires processing by the multi-catalytic protease complex, called the proteasom, in order to be presented with the MHC I complex at the cell surface of tumor cells [84]. Immunoselection may lead to tumor variants with defective antigen-processing/presenting machinery. This can be observed by the loss or down-regulation of MHC class I [81] or the down-regulation of molecules involved in the Ag-processing including transporter associated with antigen processing 1 (TAP1) or low-molecular-mass protein 2 (LMP2) [85]. Eventually this will lead to a compromised presentation of Ags, which are need in turn to be detectable by the adaptive immune system thus generating tumor cells which are poorly immunogenic.

Elimination of immune effector cells

It has been shown, that persistent triggering of the T-cell receptor can lead to a phenomenon referred to as activation-induced cell death (AICD) [86]. The tumor tissue is a massive source of Ags that eventually chronically stimulate infiltrating T cells and may become exhausted leading to AICD [87]. Further, it is a fact that tumor cells can express functional FasL and this will eventually lead to apoptosis of Fas-expressing tumor-specific T cells [88 89 90 91].

Anergy/Tolerance

Euplastic cells are antigenic, but often weakly to non-immunogenic. Basically, they express Ag against which an immune response could be directed, but lack the capacity to create a pro-inflammatory environment necessary to sustain efficient T cell activation and clonal expansion following Ag recognition. DCs have been shown to play a major role in dictating whether T cell priming or tolerance occurs [92]. For full T cell activation, a DC must not only present the Ag, but also needs to provide the T cells with relevant co-stimulatory signals, such as cell surface expression of a family of B7 molecules, as well as the production of pro-inflammatory cytokines [93] [92]. In an immunosuppressive tumor microenvironment, inflammatory mediators may be absent where DCs may fail to express the requisite pro-inflammatory and co-stimulatory characteristics that are needed to induce effector-functions in the Ag-specific T cells. This insufficient or inappropriate Ag processing and presentation may lead to energy or tolerance and lead to a poor immunogenicity of the tumor [94] [95].

Production of immunosuppressive molecules:

Malignant cells release soluble factors, such as immunosuppressive cytokines, prostaglandins and vascular endothelial growth factor, which redirect immune responses toward a favorable environment for growth. A wide variety of malignant cells locally secret IL-10. This favors the polarization towards a T_H2 response resulting in a less effective local anti-tumor immune reaction [96]. It has been shown that IL-10 production from neoplastic populations involves STAT3 activation [97] [3] [98]. Another important factor with pivotal function within the immune system is TGFß. This immunosuppressive cytokine maintains tolerance via the regulation and suppression of lymphocytes proliferation, differentiation and survival. A wide variety of malignancies produce TGFß resulting in suppression of the immune system and inversely promotion of tumor progression [99] [100].They themselves on the other hand no longer express TGFß receptors or have a malfunctioning in the TGFß signaling to circumvent the effects of TGFß on themselves [101]. It has also been shown, that tumor cells may become unresponsive to IFNγ, caused by either the absence or abnormal function of distinct components of the IFNγ receptor signaling pathway [28].

Induction of regulatory/suppressor cells

Tumors may also facilitate the generation, activation, or function of immunosuppressive T cell populations, such as type II NKT cells [102] or Treg [103] [104]. Tregs have been originally identified as a $CD4^+$ T cell subset comprising 5-10% of all peripheral T cells. They constitutively express CD25 that controls the behavior of auto reactive T cells *in vivo* and suppresses T cell responses *in vitro*. Tregs may block the activation of CTLs and block NK cell cytotoxicity. Studies in which Tregs have been depleted using an anti-CD25 mAb enabled mice to reject tumors that grew progressively in control mice [103] [105]. Furthermore, there are some studies suggesting a role of myeloid-derived-suppressor cells (MDSC) in tumor progression. MDSC arise from myeloid progenitor cells that do not terminally

differentiate into mature macrophages, DC or granulocytes (reviewed in Ostrand-Rosenberg 2010 [106]). There are two major classes, granulocytic and monocytic MDSC. Granulocytic MDSC ($Gr1^+CD11b^+Ly6G^+$) are polymorphonuclear and contain high levels of arginase, while monocytic MDSC ($Gr1^+CD11b^+ICAM\text{-}1^+F4/80^+Ly6C^+$) are mononuclear and contain both arginase and inducible nitric oxide synthase (iNOS). There are different ways how MDSC can suppress antitumor immunity. They may use a mechanism in which arginase catabolizes L-arginine. L-arginine is required for protein synthesis and by excessive use of it, MDSC basically empty their environment with leads to L-arginine deprived T cells. These T cells are deficient for the CD3ξ chain and are arrested in the G0-G1 phase of the cell cycle. In addition, granulocytic MDSC are able to release high levels of reactive oxygen species (ROS) which in turn will block T cell activation or even induce apoptosis in T cells. Monocytic MDSC on the other hand, can elevate iNOS and in turn generate NO. Further they are able to induce Tregs to disturb T cell activation.

Cancer Immunosurveillance in Humans

Given that there is significant evidence supporting the existence of a cancer immunosurveillance process in mice the question arises whether there is a similar process in humans. One would predict that immunodeficient or immunosuppressed humans should show greater incidences of cancer. And indeed, analysis of individuals with congenital or acquired immunodeficiencies or patients undergoing immunosuppressive therapy has documented a highly elevated incidence of virally induced malignancies such as Kaposi's sarcoma, non-Hodgkin's lymphoma and cancers of the anal and urogenital tracts compared with immunocompetent individuals [107-109]. However, the study of the incidence of cancers of non-viral origins that may take many years to develop is confounded by the variety of viral and bacterial infections to which these immunodeficient/immunosuppressed patients are susceptible and by the more rapid appearance of virally induced tumors. Nevertheless, one can draw upon three lines of evidence to suggest that cancer immunosurveillance indeed occurs in humans: (a) immunosuppressed transplant recipients display higher incidences of nonviral cancers than age-matched immunocompetent control populations [110 111 112] (b) cancer patients can develop spontaneous adaptive and innate immune responses to the tumors that they bear, and (c) the presence of lymphocytes within the tumor can be a positive prognostic indicator of patient survival [113, 114 115].

Skin Cancer

Cancer is generally divided into solid tumors or systemic cancer. Unlike systemic cancer diseases, solid tumors evolve from aberrant cells within an organ. This is the case for 95 percent of all cancer including skin cancer. Leukemia and lymphoma, they on the other hand evolve from the blood or lymph system and therefore affect the whole organism.

The skin is the biggest organ of the human body. It belongs to a first line of nonspecific defense and protects the inner organs from external influences like heat or pressure but also regulates the body's temperature. Composed of epithelial tissues it covers and lines our body. There are two primary layers: epidermis and dermis (**Image 3**). The epidermis is the outermost layer and contains mostly keratinocytes, which are responsible for the production of keratin. Undergoing a process called cornification they produce more and more keratin. Fully cornified keratinocytes that form the outermost layer are constantly shed off and replaced by new cells. Melanocytes are found in the bottom layer of the epidermis. They produce melanin, a pigment primarily responsible for the color of the skin and therefore block a minimal part of the UV-rays. Sometimes, clusters of melanocytes and surrounding tissue form non-cancerous growths called moles or nevi. Moles are very common. Most people have between 10 and 40 moles. The dermis is the second layer underneath the epidermis. It consists of connective tissue and cushions the body from stress and strain. Unlike the epidermis it harbors blood and lymphatic vessels, mechanoreceptors, hair follicles, sweat and sebaceous glands. The hypodermis is a third layer just underneath the dermis. Technically it does not belong to the skin but finally attaches the skin to the underlying bone and muscle, and supplies the skin with blood vessels and nerves. It consists of loose connective and adipose tissue.

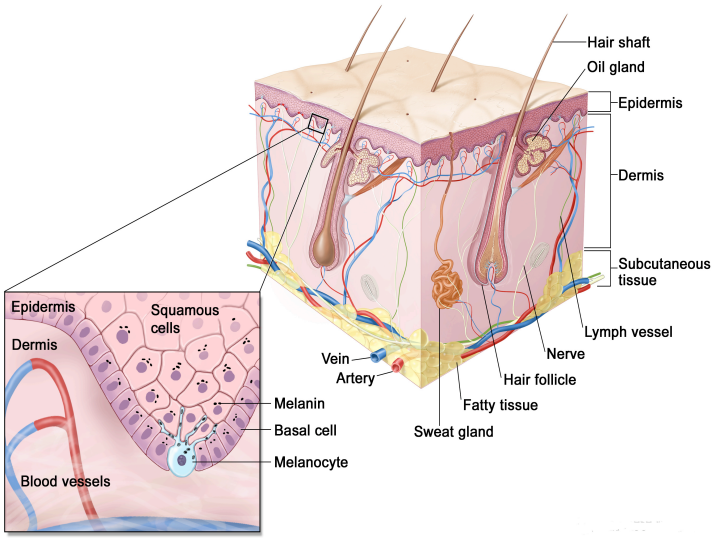

Image 3 Anatomy of the skin[116]

The Scheme shows the anatomy of the skin with the epidermis, dermis, and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis.

In Switzerland, each year about 36000 people are diagnosed with cancer. At the same time, each year 16000 people die from cancer. In terms of total deaths, this accounts for about 25 percent. One quarter of the yearly cancer incidence is due to skin cancer (excluding non-melanoma cancer) [117]. But there is a much broader variety of skin cancer depending from what cell of the skin the cancer arises. Less common skin cancers are for example Dermatofibrosarcoma protuberans, Merkel cell carcinoma and Kaposi's sarcoma and keratoacanthoma. The *Dermatofibrosarcoma protuberans* is a rare neoplasm of the dermis layer and arises from transformed connective tissue cells and is therefore classified as a sarcoma. It usually behaves as benign tumor but in some cases can metastasize. The *merkel cell carcinoma* is a rare and highly aggressive cancer. Merkel cells are oval receptor cells found in the basal layer of the epidermis. The majority of merkel cell carcinomas appear to be caused in part by a newly discovered virus the merkel cell Polyomaviurs [118]. *Kaposi's sarcoma* is a tumor caused by the human herpes virus 8 [119]. Despite of its name, it is generally not considered a true sarcoma, since it arises as a cancer of lymphatic endothelium and not from transformed connective tissue cells. *Keratoacanthoma* is a relatively common low-grade malignancy that originates in the pilosebaceous glands and closely resembles squamous cell carcinoma.

The three most common type of skin cancer however are basal cell carcinoma, squamous cell carcinoma and malignant melanoma. All of them arise from the epidermal layer of the skin [120]. *Basal cell carcinoma*, the most common type, typically it is a slow growing cancer arising from cells of the basal layer in the epidermis and hardly every metastasizes. Chronic exposure to sunlight causes most of the cases. Therefore they mostly appear on forehead, nose, ears and lower lips, places the most exposed to the sun. People who have had one basal cell carcinoma are at risk for developing others over the years, either in the same area or elsewhere on the body. *Squamous cell carcinoma* is the second most common type of skin cancer. It arises from the keratinocytes forming a squamous cell layer in the epidermis and is also considered a slow growing cancer, which in some cases may metastasize. It may occur on all areas of the body including the mucous membranes and genitals, but are most common in areas frequently exposed to the sun. The most aggressive one is the *melanoma* (also known as black melanoma). It causes the majority of skin cancer related deaths. It is a malignant tumor of the skin arising from aberrant melanocytes of the epidermis. Seldom, it can appear on eye, meninges or mucosal membranes. The melanoma is most of the time strongly pigmented. Only a few melanomas produce no or only little pigments, like the amelanotic melanoma [121].

Melanoma

Risk Factors for Melanoma

There are several factors which clearly increase the risk for developing melanoma [122] [123]. Ultraviolet radiation (UVR) is the major environmental know etiologic agent associated with melanoma. In particular intermittent sun exposure with large blasts of UVR may promote the development of melanoma [124] [125]. Further there has been mounting evidence for the negative effects of tanning beds [126] [127]. Along with the sun exposure it has become clear that the individual complexion, tanning abilities and extent of freckling may increase the risk of developing melanoma. Dysplastic Nevi however, are the strongest risk (**Image 4**). These atypical moles can be distinguished visually by clinical features of size greater than 6mm in diameter, color variegation, indistinct borders and textured surface. They are more likely to become cancerous than ordinary moles [128]. But also a higher than average number of moles shows an increased risk for melanoma [129] [130]. The risk of developing melanoma may also run in the family [131]. First-degree relative of melanoma patients have a higher risk of the disease than individuals without positive family history. This familial melanoma accounts for an estimated 5 to 10 percent of all cases of melanoma [132]. Genetics has made it possible to identify genetic factors that are critical of susceptibility to melanoma [133]. Pigmentation genes are known to be associated with melanoma, but also genetic variants in DNA repair genes are obvious candidates for melanoma susceptibility [134]. Furthermore, there have been few mutations found in cell cycle genes in the melanoma etiology [135] [136].

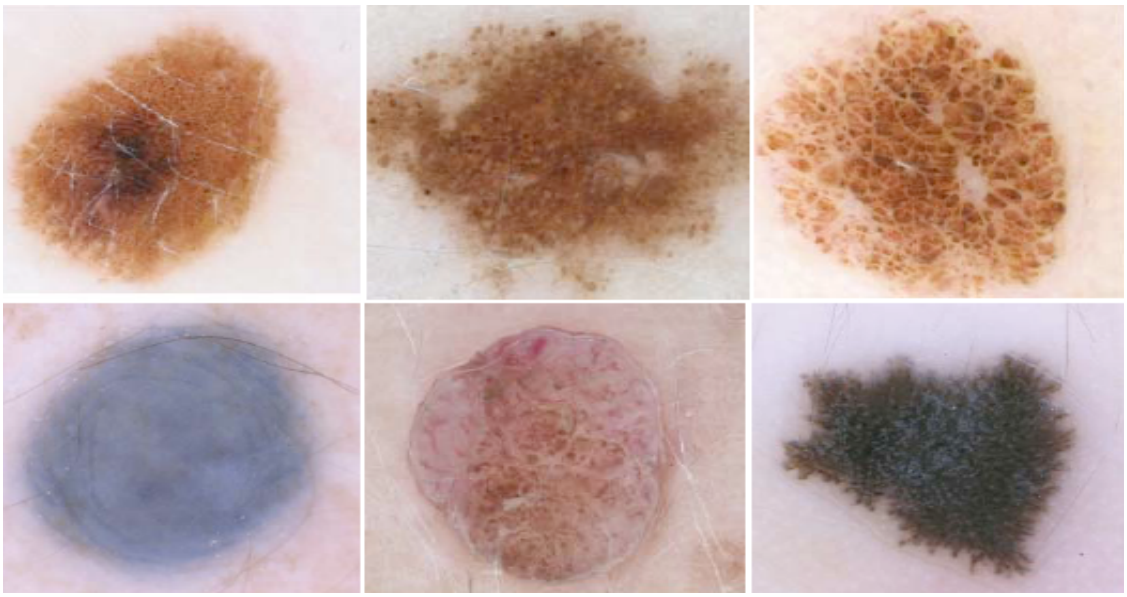

Image 4 Examples for dysplastic nevi [137]

Types of Melanoma

A melanoma can have different forms of appearance and is accordingly clinically classified (**Image 5**). The most common types are: superficially spreading melanoma, lentigo maligna, acral lentiginous melanoma and nodular melanoma [138] [139]. Each subtype has a characteristic growth pattern and develops at favored spots on the human body. *Superficial spreading melanoma* is by far the most common type and is the one most often seen in young people. As the name suggests, this melanoma travels along the top layer of the skin for a fairly long time before penetrating more deeply. The first sign is the appearance of a flat or slightly raised discolored patch that has irregular borders and is somewhat geometrical in form. The color varies between areas of tan, brown, black, red, blue or white. This type of melanoma can occur in a previously benign mole. *Lentigo maligna* is similar to the superficial spreading type, as it also remains close to the skin surface for quite a while, and usually appears as a flat or mildly elevated mottled tan, brown or dark brown discoloration. This type of melanoma is found most often in the elderly, arising on chronically sun-exposed, damaged skin on the face, ears, arms and upper trunk. When this cancer becomes invasive, it is referred to as lentigo maligna melanoma. *Acral lentiginous melanoma* also spreads superficially before penetrating more deeply. It is quite different from the others though, as it usually appears as a black or brown discoloration under the nails or on the soles of the feet or palms of the hands. *Nodular melanoma* is usually invasive at the time it is first diagnosed. The malignancy is recognized when it becomes a bump. It is usually black, but occasionally is blue, gray, white, brown, tan, red or skin tone. The most frequent locations are the trunk, legs, and arms, mainly of elderly people, as well as the scalp in men. This is the most aggressive of the melanomas, and is found in 10 to 15 percent of cases.

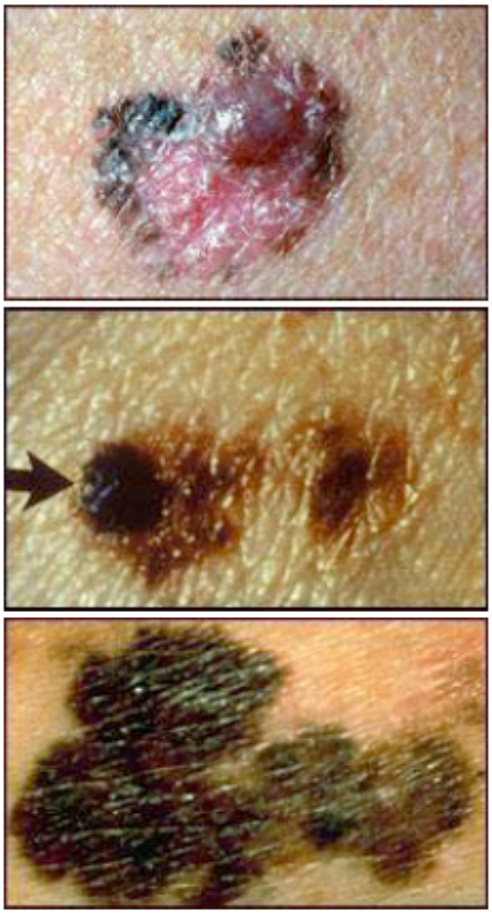

Image 5 Examples of melanoma

Stages of Melanoma

Classifications for melanomas are called stages. In the year 2001 a new American Joint committee on Cancer (AJCC) / Union Internationale Contre le Cancer (UICC) -classification and staging system for the malignant melanoma has been introduced (**Image 6**). The stage refers to the thickness (Breslow's thickness) and the presence of microscopic ulceration in the primary tumor, depth of penetration (Clark's level of invasion), and the degree to which the melanoma has spread. This staging is usually used to determine treatment. While early melanomas (Stages I and II) are localized, more advanced melanomas (Stages III and IV) have metastasized to other parts of the body [140] [141] [142].

Stage I	This category is subdivided according to the thickness of the primary (original) tumor.	
	Stage Ia	The tumor is less than 1.0 mm in Breslow's thickness without ulceration and is in Clark's level II or III.
	Stage Ib	The tumor is less than 1.0 mm in Breslow's thickness with ulceration and/or Clark's level III or IV, or it is 1.01 - 2.0 mm in thickness without ulceration, and may have spread to the closest lymph nodes.
Stage II	This is also subdivided according to gradations in thickness and/or depth, the presence or absence of ulceration, and potential regional lymph node metastases.	
	Stage IIa	The tumor is 1.01 - 2.0 mm in Breslow's thickness with ulceration, or is 2.01-4.0 mm in thickness without ulceration.
	Stage IIb	The tumor is 2.01-4.0 mm in Breslow's thickness with ulceration, or is greater than 4.0 mm in thickness without ulceration.
	Stage IIc	The tumor is greater than 4.0 mm in Breslow's thickness with ulceration.
Stage III	Once a melanoma is known to have reached the local or regional lymph nodes, the disease is said to have reached Stage III. Breslow's thickness is no longer used in staging, but the presence of microscopic ulceration continues to be used, as it has an important effect on the progression of the disease. In-transit or satellite metastases are also included in Stage III. In this case, the spread is to skin or underlying tissue (subcutaneous) for a distance of more than 2 centimeters (1 cm equals 0.4 inch) from the primary tumor, but not beyond the regional lymph nodes. In addition, the new staging system includes metastases so tiny they can be seen only through the microscope.	
Stage IV	The melanoma has metastasized to lymph nodes far away from the primary tumor or to internal organs, most often the lungs, followed in descending order of frequency by the liver, brain, bone and gastrointestinal tract.	

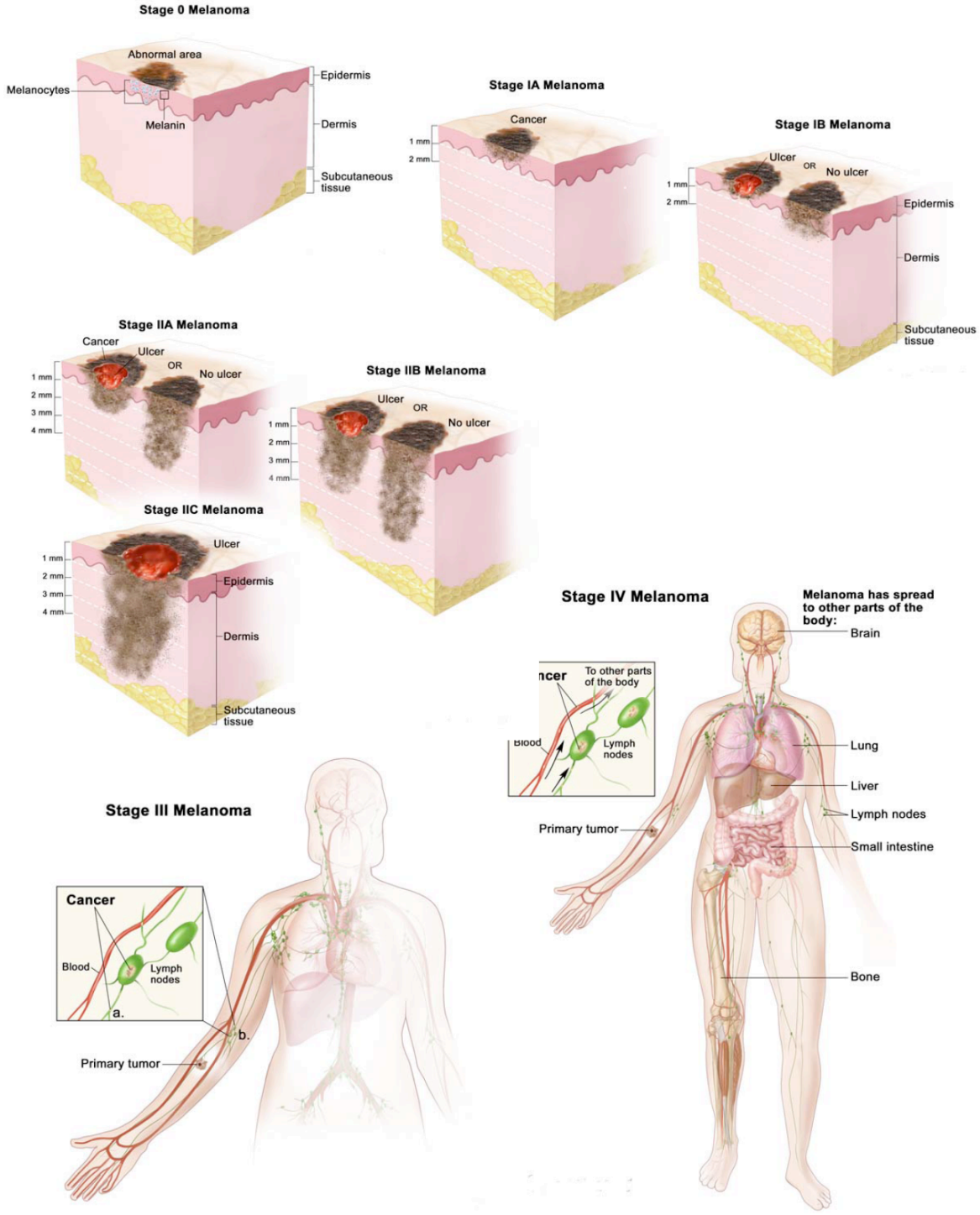

Image 6 Melanoma stages [143]

Melanoma Treatment

Surgery to remove the tumor is the primary treatment of all stages of melanoma. And early detection still remains the best weapon in fighting skin cancer. Most people with thin, localized melanomas are cured by appropriate surgery excision [144]. In addition, lymph nodes may be removed to examine eventual cancer spreading. Further treatment greatly depends on the diagnosed stage of the melanoma and includes the following treatments:

Chemotherapy: A number of drugs that are active in fighting cancer cells are being used to treat melanoma, either one at a time or in combinations. They impair cell division, which results in the death of rapidly dividing cells. Currently, the best single agents include Dacarbazine (DTIC), cisplatin and other alkylating agents [145]. DTIC is however, the only chemotherapeutic agent approved by the Food and Drug Administration (FDA) for melanoma treatment. Treatment with DTIC (usually i.v.) leads to a response rate of 15-25 percent, with median response durations of 5-6 month, but less than 5 percent of complete responses [146]. Common toxicities include mild nausea and vomiting, myelosuppression and fatigue. Another drug, Temozolomide (TMZ), an analog of DTIC, can be given orally and has the advantage to penetrate the CNS and may prevent melanoma brain metastases. In terms of response rate and overall survival, TMZ did not show an increase compared to DTIC. TMZ however, is very well tolerated and shows an advantage in terms of improvement in the quality of life. The isolation-perfusion method is sometimes used as a pain-relieving treatment when the melanoma is on an arm or leg. Unfortunately, to date, the response of melanomas to chemotherapy has been limited.

Radiation therapy: This cancer treatment uses high-energy x-rays or other types or radiation to kill cancer cell or keep them from growing. There are two different application types, the external radiation therapy or the internal radiation therapy depending on the stage and type of the cancer being treated. Even tough, melanoma is considered a relatively radio resistant tumor, patients may derive clinical benefit from radiation of symptomatic metastases [147].

Immunotherapy: Malignant melanoma is one of the most immunogenic tumors in man; this makes it an attractive candidate for immunology-based anti-neoplastic cytokine-therapies. INFα is one of the few systemic drugs with FDA approval. IFNα has been well demonstrated to have anti-proliferative and immunomodulatory effects, including the inhibition of angiogenesis, the increase of MHC I Ag expression and the infiltration of CD4+ T cells into melanomas [146]. Indeed, high-dose interferon has been shown to increase relapse-free survival and overall survival (OS) [148] [149] [150], this however not without substantial side effects which adversely affect the patient quality of live. Adjuvant therapy with lower dose of interferon have not been consistently shown to have an impact on either relapse-free survival or OS [151]. Stage IV patients are rarely curable with standard therapy, though high-dose Interleukin-2 has been reported to produce durable responses in a small number of patients [152] [153] [154]. IL-2, has been described to play a central role in immune regulation. It has been shown to act as T cell growth factor but is also capable to activate NK and B cells as well as macrophages and further induces the production of other cytokines such as IFNγ or TNF. In patients with systemic metastasis confined to one anatomic site, long-term survival is occasionally achieved by complete resection of all metastatic disease [155] [156] [157] [158]. However, interleukin-2 is associated with very significant side effects

when given in high doses [146] and requires hospitalization with intensive monitoring. Major toxicities include fever, chills, hypotension, increases capillary permeability, cardiac arrhythmias, oliguria, volume overload, delirium and rash. Combination of cytotoxic agents with IFNα and/or IL-2 in biochemotherapies however, has demonstrated promising antitumor activity.

There are a variety of new treatments being tested in mouse models as well as in human trials:

Vaccine therapies: The vaccines are intended to stimulate the immune system so that it reacts more strongly against a patient's melanoma cells, destroying the cancer or slowing the progression. These strategies include vaccination with peptide, DCs, nucleic acids and het shock protein complexes.

Adoptive cell therapy (ACT): ACT involves the harvesting of lymphocytes from the patient, in vitro selection/expansion/activation and subsequent infusion of processed lymphocytes back into the patient to induce an immune response against the cancer cells [147].

Monoclonal antibody therapy (Targeted therapy): One option is the treatment with Anti-cytotoxic T-lymphocyte Ag (CTLA)-4 antibodies (ipilimumab and tremelimumab). CTLA-4 is expressed on activated T cells and acts as negative regulator of T cell activation and Ab against CTLA-4 may potentiate immune responses against cancer cells [147].

Anti angiogenic therapy: They are anti-angiogenic, which means that they prevent new blood vessels from forming and therefore cut off the blood supply that would otherwise nourish the cancer cells and enable them to grow. These drugs are still experimental and a good deal of research into improving and combining them with others is going on. Studies are underway with the anti-angiogenic drug, thalidomide, combined with the chemotherapeutic agent, temozolomide. Angiostatin and endostatin are two other drugs in this class that have shown some degree of activity against melanoma in preliminary studies

B16 – a Mouse Model for Melanoma

Several syngeneic transplantation models for melanoma exist in common mouse strains, including the Harding-Passey Melanoma in BALB/cxDBA/2F1 mice [159] or the Cloudman S91 melanoma in DBA/2 mice [160]. The most frequently used syngeneic murine melanoma model is however B16 derived from a spontaneously arising melanoma of C57BL/6J origin [161]. It is regarded as a poorly immunogenic model, since B16 melanoma expresses rather low level of MHC I class molecules therefore hampering the recognition by CTLs. Nevertheless, B16 melanoma cells express some melanoma-associated Ags such as Tyrosinase-related protein-2 (TRP-2) or gp100 and immunotherapeutic interventions (including cytokines) could induce tumor regression suggesting some immunogenicity of B16 tumors [162] [163]. One of the thoroughly tested cytokines within the B16 model is IL-12.

Interleukin-12

The cytokine interleukin 12 (IL-12) was originally identified as a NK cell–stimulatory factor [165] and is produced mainly by activated antigen-presenting cells [166] particular by DCs and macrophages, although it can also be secreted by PMNs. IL-12 is a heterodimeric cytokine of 70 kDa consisting of heavy (p40) and light (p35) subunits, which are covalently linked (**Image 7**) [167] [168] [169]. While p40 appears to be produced in abundance in these cell types, the production of p35 is ubiquitously and constitutively expressed only at low levels and it requires p40 co-expression for secretion of the biologically active cytokine from the cell [170]. IL-12 is mostly secreted upon TLR activation and it therefore acts as an early pro-inflammatory cytokine in response to many infectious agents [171]. The most physiologically important IL-12 targets are conventional NK (cNK) cells, NKT cells and T cells, in which IL-12 induces proliferation, the expression of cytotoxic mediators, and the production of cytokines, most importantly IFNγ, but also GM-CSF, TNF and IL-2 that are necessary for the ensuing immune response; it drives further production of IL-12 from DCs and macrophages in a positive feedback loop and finally it drives the differentiation of naïve T cells to an IFNγ-producing T_H1 effector cell phenotype [172]. Thus, IFNγ is an important mediator of the effects of IL-12. Furthermore, IL-12 favors the differentiation toward T_H1 cells [164], which are thought to yield superior antitumor immunity [71]. IL-12 signals through the heterodimeric IL-12 receptor (IL-12R) that is formed by IL-12Rβ1 and IL-12Rβ2 subunits and which predominantly bind to p40 and p35 chains, respectively [173]. Co-expression of both subunits is required for the generation of high affinity binding sites for p70 binding and IL-12Rβ2 is the critical signal transducing subunit of the receptor complex [174]. Triggering of the receptor activates the JAK-STAT signaling pathway, with STAT4 being the predominant mediator of cellular responses activated by IL-12. The IL-12R is predominantly found on NK cells and activated T cells but it has also been demonstrated on the surface of APCs implying an autocrine effect [175] [176]. The expression of IL-12Rβ2 on T_H1 cells is tightly regulated with its expression being maintained by IFN-γ and T-bet expression and inhibited by the T_H2 cytokine IL-4.

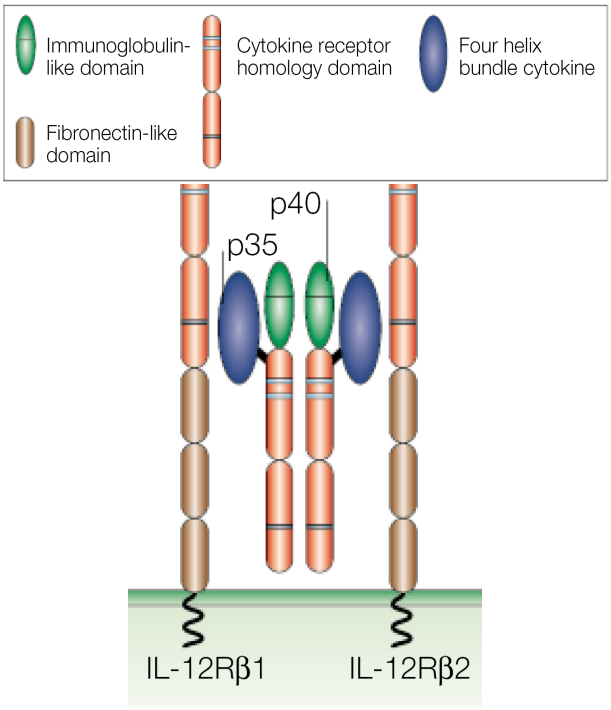

Image 7 IL-12 Signaling-Complex [164]

Interleukin-12 in Tumor Immunology

It is held that an array of different immune cells interacts with tumor cells from the earliest stages of malignant transformation to the terminal phase of widespread metastasis. These immune-tumor interactions are largely controlled and guided by cytokines produced by leukocytes. Several studies have shown that exogenously administered IL-12 impairs the growth of different tumors in vivo in natural as well as in therapeutic conditions [177] [178] [179]. Boggio *et al.* for instant showed that in transgenic mice expressing the rat HER-2/neu oncogene under the transcriptional control of mouse mammary tumor virus (MMTV) showed spontaneous tumors in mammary glands. HER-2/neu oncogene is also expressed in a substantial proportion of human mammary carcinomas. The application of IL-12 in these mice was able to prevent tumor growth [180]. Another model for spontaneously arising tumors is the SV11 mouse. These SV40 large T Ag transgenic mice show spontaneous tumor development in the choroid plexus in the brain. Again, IL-12 treatments lead to more slowly growing tumors than in control mice and in some cases tumors were even reduced in size [181]. Noguchi *et al.* showed that the IL-12 anti-tumor effect also held true in the subcutaneously MCA induced tumor. IL-12 again was able to delay tumor appearance and depending on the dose of IL-12 and MCA, also reduced tumor incidence [182]. Mice lacking the IL-12 specific subunit p35 developed significantly increased numbers of papillomas compared to wild type mice. And MCA-induced fibrosarcomata in *Il12p40*$^{-/-}$ mice showed significantly faster tumor appearance in about twice as many mice than in wt mice [50]. Also, the B16 melanoma tumor volume of mice lacking IL-12Rβ2, which specifically binds to p35, is increased compared to the tumor volume of wild type mice, supporting the notion that IL-12 represses tumor growth under normal conditions [183]. The anti-tumor activity of IL-12 is however very complex and uses effector mechanisms of both innate resistance and adaptive immunity. The host of the data in the field suggests that adaptive immune responses mediated by IL-12 induced T_H1 cells are responsible for the elimination of tumors and suggest that IL-12 lowers the threshold for T cell activation to recognize tumor Ag. Nevertheless, apart from this widely held notion, there is a body of literature, which suggests that specific recognition of tumor Ags might not always be required for the effect of IL-12 [184]. Further, this finding is contradicted by Kodama *et al.* [185], who showed that IL-12 still represses growth of B16 melanoma cells in *Rag2*$^{-/-}$ mice, lacking T cells. Also Boggio *et al.* showed that in the spontaneous tumor model of rat HER-2/neu oncogene expressing mice, IL-12 administration was still able to prevent tumor growth, even when mice were thymectomized at 4 weeks and treated with anti-CD8 mAb [180]. The seminal work by Smyth and colleagues demonstrates that NK and NKT cells contribute to the natural protection from tumor metastasis in the B16 model [31]. In *Jα281-/-* mice, which lack the invariant Vα14Jα281 TCR and therefore lack Vα14 NKT cells exclusively, IL-12 application could no longer suppress metastasized B16 in the liver after intra splenic injection compared to wt mice. In contrast, in *Rag-/-Vα14NKT* mice, which exclusively generate Vα14 NKT cells, IL-12 treatment lead to fully rejected metastasis. Similar results were obtained from B16 subcutaneously injected [186]. Supporting these findings, Nakagawa *et al.* showed, that αGalCer, the specific ligand for the Vα14Jα281 NKT cells, as well as its synthetic homologue KRN7000, proofed to be a natural inducer of IL-12 and has the ability to induce anti-tumor immunity in mice [187]. Tallying these findings, KRN700 in C26 hepatic metastases clearly showed that αGalCer activity is IL-12 dependent since it is

abrogated in *Il12p40-/-* mice [188]. The experiments of Kodoma *et al.* with *Rag2-/-* mice, lacking not only T cells but also NKT cells, did not support a role of NKT cells in IL-12 anti-tumor activity. They on the other hand propose that conventional NK (cNK) cells are critical, since the IL12-inhibited lung metastasis effect in *Rag2-/-* mice was abolished when NK cells were depleted. Further, the IL-12 effect was abrogated by perforin deficiency but not by FAS ligand deficiency [185]. This discrepancy seemed to depend on the dose of administered IL-12. While high-dose IL-12 seemed to mediated anti-tumor activity by NK cells and perforin, low dose of IL-12 suggested a partial role for NKT cells [31]. And to make it even more complex there were studies suggesting, that other γc-dependent non-T non-B cells, possibly lymphoid DCs, are activated by the IL-12-mediated rejection of subcutaneous tumors, since NK cell depletion in *Rag-/-* mice did not alter the IL-12 mediated effect [189]. Watkins *et al.* even showed that IL-12 was able to reprogram the functional profile of tumor-associated macrophages (TAMs) that had been chronically polarized in their function by the tumor environment of Lewis Lung Carcinoma (LLC) [40]. It was suggested that the site of implantation of the tumor critically affects the nature of the cell types recruited by IL-12. NK cells seemed to be involved in the IL-12-mediated rejection of liver metastases, whereas other γc-dependent probably DCs, were required for rejection of skin tumors [189]. In spite of the fact that most of the above mentioned studies use the same tumor model, namely growth of B16 melanoma cell lines under the skin or metastasis formation of these cells in the lung and liver, they each propose a different mechanism of how IL-12 represses tumor growth in mice.

IL-12 in Cancer Therapy

The effective treatment of tumor and its metastases is a challenge for any cancer treatment. Many have clearly demonstrated that IL-12 is an effective and promising antitumor cytokine. Unfortunately, preclinical and clinical trials using systemic administration of recombinant IL-12 demonstrated severe adverse side effects and only to some extend tumor regression. Early studies tried to evaluate the safety of subcutaneously (s.c.) and intravenously (i.v.) injected IL-12. A phase I trial with i.v. injection of IL-12 was performed on patients with advanced malignancies including renal cancer, melanoma and colon cancer. IL-12 (500ng/kg dose level = maximum tolerated dose (MTD)) was first administered once as an inpatient. After 2 weeks of rest, the patient received one injection daily for 5 days in every 3 weeks as an outpatient. Injection was followed by common toxicities including fever /chills, fatigue, nausea, vomiting, and headache. Furthermore, anemia, neutropenia, lymphopenia, hyperglycemia, thrombocytopenia, and hypoalbuminemia were observed. Unfortunately there was only a partial response to IL-12 (renal cancer) and one transient complete response (melanoma) [190]. The phase II trial with i.v. administered IL-12 (500nk/kg dose level) based on the phase I trial by Atkins *et al.* however resulted in severe toxicities. Of the 17 patients receiving IL-12, 12 patients were hospitalized and 2 patients died [191]. Another Phase I trial with i.v. injected IL-12 twice weekly, some partial clinical responses and stable disease were observed in patients with metastatic renal cell cancer or malignant melanoma [192]. The clinical trial of Hurteau *et al.* tried to use a pre-dosing approach to limit the toxicity. Patients with recurrent or refractory ovarian cancer received i.v. injected IL-12 (250ng/kg) as a single dose on day 1, followed by a 2-week rest period and subsequent cycles of daily administration for 5 days with 16 days rest in between the cycles. One patient was a partial responder and 13 patients out of 26 had stable disease. 21percent of the patients developed grade 4 myelotoxicity while two patients had capillary leak syndrome [193]. Early studies from Bajetta *et al.* [194] evaluated the safety profile of s.c. administered recombinant human IL-12 (rHuIL-12) and its antitumor effect in metastatic melanoma. They showed that the IL-12 treatment was tolerated, albeit again not without toxicity consisted of a flu-like syndrome including fever, chills, fatigue and arthromyalgias and some patient specific abnormalities concerning pulmonary toxicity, hyperfibrinogenemia and anemia. The fixed dose given at three time points during two identical 28-day cycles lead to tumor shrinkage involving regression of s.c. nodules in 3 out of 10 patients. However, while circulating IL-12 peaked 8-12 h after the first injection, levels dropped below detectable values 24-30 h after drug administration, therefore the repeated administration. This repeated administration of rHuIL-12 however lead to an anti-IL-12 immune response that progressively inhibited the diffusion of rHuIL-12 into the bloodstream or lead to more rapid clearance of rHuIL-12 from the blood. This unprecedented phenomenon could not be explained on the basis of an antibody response to the injected cytokine. Phase I studies of s.c. administration of IL-12 by Motzer *et al.* and Portielje *et al.* in patients with advanced renal cancer resulted in more limited toxic effects. Escalating doses of IL-12 given on day 1,8,15 of a 28-day cycle showed dose-limiting toxicities like increase in transaminase concentration, pulmonary toxicity, and leukopenia. The most severe toxicities occurred with the first injection and were milder upon further treatment. [195]. The pre-dosing protocol of Portielje *et al.* relied on a single IL-12 dose followed by a

three times weekly s.c. injection after 8 days of rest. Dose-limiting toxicity comprised deterioration of performance status, fever, vomiting, mental depression, and leukopenia. In comparison with a single administration, the three times weekly administrations at the same dose level were accompanied with a milder pattern of side effects [196]. The use of gene therapy for the delivery of the cytokine was therefore the next focus of research to improve the antitumor treatment with IL-12. Several methods for the delivery of IL-12 have been tested, some of them more successful than others. A newer study evaluated the safety and efficacy of intratumoral application of plasmid DNA encoding IL-12 with patients bearing malignant melanoma with stage IV [197]. The application of IL-12 DNA (three injections per cycle, for up to seven cycles) induced clinical responses, which lead to one complete remission and two stable diseases in 9 patients. Furthermore, they could detect a local response at the injection site in all but one of the 9 patients. An additional study confirmed these results of local intratumoral injection of IL-12 plasmid, however, showed that no change was seen in non-treated distant lesions [198].

Rakhmilevich *et al.* administered an IL-12 cDNA gene construct *in situ* into skin tissue via gene gun delivery which resulted in minimal side effects but conversely did not lead to an improved tumor regression [199]. A phase I trials of IL-12 gene therapy used genetically engineered autologous fibroblasts in patients with advanced malignancies. Primary fibroblast cultures from patients were transduced using a retroviral vector carrying the huIL-12 gene. Out of 13 patients they reported 2 reductions of tumor sizes at injection site and 2 reductions of tumor sizes at non-injection sites [200] [201]. Another treatment of tumor-bearing BALB/c mice relied on a single intratumoral injection of biodegradable polylactic acid microspheres loaded with recombinant IL-12 which promoted complete regression of the primary tumor and prevented the metastatic spread to the lung.[202]. The injection however was done at a very early time point of the tumor growth and was not shown to work also in later stages. Approaches with in vivo electroporation of IL-12 as gene delivery technique have also been tested in the B16 tumor model. The intratumoral application of a plasmid encoding IL-12 with following in vivo electroporations resulted in a cure of 47 percent of tumor bearing mice. Further, 70 percent of the cured mice were resistant to challenge with B16 [203, 204]. A further method to deliver IL-12 is viral-mediated. Oncolytic adenoviruses have been developed as anticancer biological agents [205]. These conditionally replicating adenoviruses (CRAds) specifically replicate in cancer cells and kill these. Within a solid tumor mass for instance, the release of newly formed infectious particles from infected cancer cells allows additional cell layers to be infected and destroyed. Tough used as monotherapeutic agents, CRAds showed only limited effects in clinical trials. These CRAds have been used by *Lee et al.* to deliver IL-12 and elicit a potent antitumor effect in the B16-F10 murine melanoma model [206]. When intratumoral injected into tumors of the size around 80 to 100mm^3, tumor growth was significantly reduced compared to the PBS injected control group. Given that the mode of action of IL-12-mediated tumor suppression has yet to be discovered.

Lymphoid Tissue inducer cells

Fetal Lymphoid Tissue inducer cells

In 1992, Kelly and Scollay discovered in studies with newborn mice, the accumulation of a new cell population positive for CD4 but negative for CD3 [207] [208]. Being present in high numbers in the neonatal LN, this population did not seem to increase greatly after birth but instead rapidly diminished in proportion to $CD3^+$ cell numbers. The cells seemed to have a lymphoid morphology, but their origin and function have not been determined at that time. Some years later, Mebius *et al.* [209] clearly showed that these $CD4^+CD3^-$ cells uniformly express CD45 and thus belong to the hematopoietic system. However, they neither rearranged the ß chain of the TCR nor the IgH chain, and did not express pTα nor had detectable mRNA levels for the recombinase enzymes RAG1 and RAG2, suggesting that they do not belong to the T or B lymphocyte-rearranging subsets. Instead, *in vitro* studies suggested that this cell type in the presence of IL-2 had the ability to express the NK cell marker Nk1.1 and in response to IL-4 and GM-CSF showed potential APC skills in mixed lymphocyte reactions (MLR). In addition, $CD4^+CD3^-$ cells expressed LTαß, IL-7Rα and IL-2Rα at high levels. About 75 percent of the cells further expressed IL-2Rα (CD25) and MHC II. This cell type could be found in fetal/neonatal lymph nodes, spleen and intestine but not in fetal liver or thymus. Mebius and others proposed that $CD4^+CD3^-$ cells play a role in the initiation of lymphoid structure during ontogeny [209] [210] [211], which would tally with the findings of null mutations of LTα, LTß, LTßR, IL7Rα and IL2Rα chains resulting in the lack of LNs [212] [213] [214] [215] [216] [217] [218] [219]. Finally, two experimental approaches have demonstrated that $CD4^+CD3^-$ cells clearly play a role in the initiation of lymphoid structures and were therefore termed 'lymphoid tissue inducer' (LTi) cells. Firstly, *CXCR5*$^{-/-}$ mice have been shown to have drastically reduced numbers of Peyers Patches (PP). But the PP development was restored when fetal wild type $CD4^+CD3^-$ cells were transferred into newborn *CXCR5*$^{-/-}$ mice [220]. Furthermore, *Id2*$^{-/-}$ mice completely lack nasopharyngeal-associated lymphoid tissue (NALT). And again, adoptive transfer experiment of fetal intestine $CD4^+$ $CD3^-$ cells into *Id2*$^{-/-}$ newborn mice let to the initiation of NALT-like structures [221]. Secondly, mice lacking $CD4^+CD3^-$ cells have been shown to completely lack LNs and PPs [222] [223].

The phenotype of LTi cells has been carefully described [209] [211]. They express ligands of the tumor necrosis factor (TNF) family such as LTαß, TRANCE, LIGHT and TNFα, the chemokine receptors CXCR5 and CCR7, the adhesion molecules Integrin α4ß1, Integrin α4ß7 and ICAM-1. Moreover these cells are positive for CD25 (IL-2Rα), CD44, CD90.2 (Thy1.2), CD122, CD117 (c-Kit) and CD132 (γc). Not all LTi cells seem to express CD4. But other than this exception, the two subsets exist with virtually identical surface markers and quantitative patterns of gene expression [224]. The proportion of $CD4^+$ and $CD4^-$ LTi cells seems to vary depending on the different LNs being analyzed [224]. Functional experiments with $CD4^-$ cells have not been done and it remains to be tested if they really do belong to the LTi cell pool.

Table 1 Phenotypic comparison of human and mouse LTi cells [225]

Protein	Mouse	Human	mRNA	Mouse	Human
CD127	+	+	*RORC*	+	+
CD4	+	–	*LTA*	+	+
CD3ic	–	–	*LTB*	+	+
CD5	–	– to low	*TNFSF11*	+	+
CD7	ND	50–70%	*TNFRSF11*	+	+
CD10	ND	–	*ID2*	+	+
CD16	ND	–	*TCF3*	+	+
CD33	ND	–	*RAG2*	–	–
CD34	ND	–	*CCR7*	+	+
CD44	+	+			
CD45	Int	Int			
CD54	+	+			
CD94	ND	–			
CD62L	–	–			
CD117	– to low	low			
CD161	ND	40–50%			
CXCR5	+	80%			
TdT	–	–			

CD3ic, intracellular CD3; ND, not determined; Int, intermediate; %, percent cells expressing marker; TdT, terminal deoxynucleotidyl transferase; RAG2, recombination-activating gene 2. Data are representative of five experiments.

Two potential progenitor of LTi cells have been described so far in fetal liver [226] [227]. Akashi and coworkers have characterized IL-7Rα^{+}Sca-1^{low}c-Kit^{low} cells in the fetal liver with the ability to give rise to CD45^{+}CD4^{+}CD3^{-} cells both *in vivo* and *in vitro*. And Yoshida *et al.* described a lin^{-}IL-7Rα^{+}α4ß7^{+} cell population that could differentiate into CD45^{+}CD4^{+}CD3^{-} cells. The IL7Rα surface expression in the absence of lineage markers is a common feature these LTi progenitors share with lymphoid progenitors. However, the two progenitors must diverge at some earlier stage, since the LTi progenitor lost its B-cell potential with the expression of the integrin α4ß7^{+} [227]. This differentiation of progenitors into LTi cells requires the presence of the transcription factors Ikaros [228] and nuclear retinoic acid related-orphan receptor RORγt [223]. The Ikaros gene encodes a family of early hematopoietic- and lymphocyte-restricted transcription factors. Mice with homozygous mutation in the Ikaros gene have only a rudimentary thymus with no definitive T cell precursors and furthermore lack LNs and PPs [228]. As expected, RORγt deficient mice lack LTi cells and do not develop LNs and PPs. RORγt is also expressed by pro-inflammatory IL-17^{+} T helper cells and further promotes survival of double positive thymocytes. Latter might lead to the abnormal thymus in RORγt deficient mice [223] [229] [230]. LTi development further depends on the protein Id2, since Id2 deficiency comes along with the absence of LTi cells and thus leads to the lack of LNs, PPs and NALT. Id proteins are inhibitors of basic helix-loop-helix transcription factors and play an important role in the regulation of lineage commitment and differentiation [222]. LTi cells have been shown to co-express TNF-related activation-induced chemokine (TRANCE; also known as RANKL) and its receptor TRANCER (also known as RANK) [231]. It has been speculated that TRANCE mediates the differentiation of precursors towards LTi cells at the locations of LN organogenesis since in the absence of TRANCER signaling, LTi cells in rudimentary LNs are markedly reduced in numbers [231] [232] [233].

Human mesentery in the first trimester and developing human LNs in the second trimester contained a population of Lin^{-}CD127^{+}RORγt^{+}CD4^{-} cells [225]. Spits and coworkers found that these cells showed a similar phenotype as the LTi cell type in mice and were therefore considered to be its human counterpart. They further showed that the co-culturing of fetal human mesenchymal stem cells (MSC) with LN derived Lin^{-}CD127^{+}RORγt^{+} cells indeed induced VCAM1 expression in a TNF and LTß dependent manner [225].

The Role of Lymphoid Tissue inducer Cells in Lymph Node Organogenesis

During embryogenesis, endothelial-cells bud from larger veins and form so called lymph sacs. Within these lymph sacs, clustering of IL-7Rα^{+} cells (inducer) with VCAM-1^{+} resident stromal cells (organizer) takes place during the early formation of LN *anlagen* (E12.5 and E13.5). This interaction between inducer and organizer cells is mediated by activated integrin α4ß1 [220]. Stimulation of the IL-7R pathway induces the expression of LTα1ß2 in LTi cells [234] which in turn eventually allows LTßR triggering on stromal cells. Studies in KO mice have shown that molecules of the TNF super-family are crucial for the development of LNs. Indeed, in the absence of LTα, LTß or LTßR, LN development is blocked, indicating that the engagement of LTßR is an essential step for LN organogenesis. Additional, injection of a LTßR agonist antibody (Ab) into LTα$^{-/-}$ mice is able to restore LN development. The stimulation of LTßR activates two NFκB pathways where as the alternative trail will lead to chemokine production, such as CCL19, CCL21, CXCL12 and CXCL13, generally important to attract T and B cells [235] [236] [237]. CXCL13 binds to CXCR5, the corresponding chemokine receptor expressed by LTi cells, and leads to the activation of integrin α4ß1 [220].The activated form of α4ß1 binds to VCAM-1, thereby leading to a firm adhesion between LTi cells and organizer cells. Since mice deficient for CXCR5 or CXCL13 lack most LNs, it is clear that chemokine/chemokine receptor family members are required for LN development. The classical NFκB pathway will lead to further up regulation of VCAM1 expression on organizer cells and in turn causes the accumulation of more LTi cells and therefore a positive feedback loop on the LTßR signaling [238]. Eventually a large enough cluster develops, and blood vessels start to differentiate into high endothelial venules (HEVs), which allow cells to enter from the bloodstream. Subsequently, organization into distinct B- and T- cell areas occurs in these organs (**Image 8**) (reviewed in Mebius 2003 [239]).

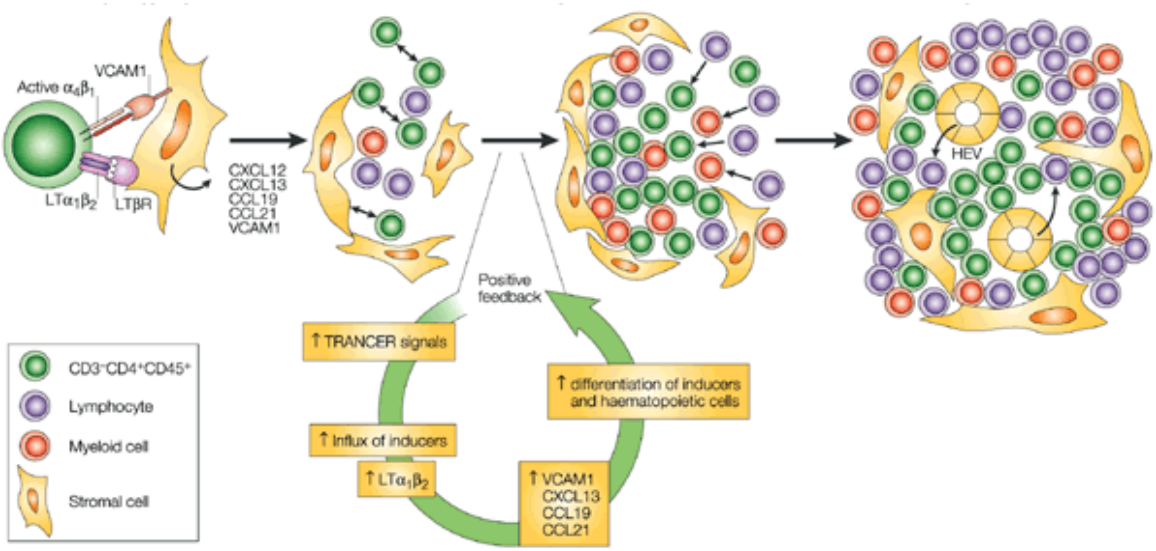

Image 8 Model for lymphoid-organ development [239]

Interleukin 7

IL-7 is a member of the IL-2/IL-15 cytokine family and was originally discovered as a result of its proliferative activity on immature murine B cells *in vitro* [240]. It is expressed by mesenchymal and epithelial cells in the gut, bone marrow (BM) and thymus and can also be expressed by fibroblasts, smooth muscle cells, keratinocytes and DCs following activation [241] [242]. The IL-7 receptor (IL-7R) is composed of the common cytokine gamma chain (γc) and the IL-7Rα chain [240] [241]. In mice, expression of the IL-7Rα chain is limited to hematopoietic cells, mostly from the lymphoid lineage. Both IL-7Rα and γc subunits are expressed by LTi cells. [241] [242]. IL-7 has been implicated in B and T cell development and in γδ TCR rearrangement. Further, IL-7 plays a role in survival/persistence of resting CD4 and CD8 T cells and their homeostatic turnover. It may also be responsible for the differentiation of effector to memory T cells [243] [244]. IL-7-deficient mice, have a blocked B cell development at the pro-B cell stage, the thymic cellularity is decreased 20-fold, have a reduced αß T cell population, whereas γδ T cells are nearly absent [243]. Lacking IL-7, IL-7Rα or the signaling component of IL-7R, such as Jak3 results in severe defects in LN and PP organogenesis in mice [245] [246] [216] [247]. Thus, IL-7 is important during lymphoid organogenesis. Indeed, IL-7 produced by fetal organizer cells has a key function in inducing LTαß expression by LTi cells [248] [245].

Adult Lymphoid Tissue inducer Cells

LTi cells involved in the LN organogenesis obviously can be found in neonatal mice. The question arises though, whether these cells are still present in adult mice. Several studies have addressed this issue in the past years. However, the first study was published as early as 1993 and focused rather on helminths infection than adult LTi cells. Nevertheless, Estes *et al.* showed that upon parasite infection with Mesocestoides corti, enriched $CD4^+$ splenocytes from infected animals and cultured in the presence of the parasite, increase in terms of $CD4^+CD3^-$ cells while $CD4^+TCR\alpha\beta^+$ cells seemed to decline. In addition, flow cytometry analysis of spleen and liver of infected mice revealed an increased number of $CD4^+CD3^-$ cells compared to uninfected mice. It has been hypothesized that these cells may play a role in maintaining the balance between host and parasite [249]. Almost 10 years later, Littman and coworkers who already proofed the requirement for RORγt in fetal LTi cells [223], showed in adult mice clusters of $ROR\gamma t^+$ cells within Cryptopatches (CPs) and to lesser extent in isolated lymphoid follicles (ILFs) and subepithelial dome of PPs. These cells proofed to be c-kit and IL-7Rá positive but negative for all lineage markers except CD4. These adult intestinal RORyt cells share developmental, phenotypic and functional features with fetal LTi cells [230], [250]. Indeed, a recent study showed that the reconstitution of $ROR\gamma t^{-/-}$ mice with bone marrow (BM) as a source for $ROR\gamma t^+$ LTi cells from adult mice induced the development of organized structures in the gut [251]. Also the transfer of wild type BM into LTα-/- or γc-/- mice allowed the reconstitution of CPs and ILFs, thus BM may be a source of LTi-like precursors [250] [251]. Adult LTi cells have also shown to be present in the spleen; more precise in B follicles and at the interface between the B and T cell area [252]. The data showed that the cells here are closely associated with Ag-specific T cells during primary and memory phase of immune responses. High levels of OX40L (also known as TNFSF4) and CD30L (also known as TNFSF8) expression by $CD4^+CD3^-$ cells potentially provides co stimulatory signals to primed T cells and may ensure that T cells are fully committed to the provision of B cell help. Adult but not fetal LTi cells exclusively expressed these TNF ligands for OX40 and CD30 after *in vitro* culture. However, addition of IL-7 to neonatal LTi cells increased CD30L expression [253]. This lack of OX40L and CD30L expression on neonatal inducer cells could be a contributory mechanism for neonatal tolerance induction [253]. Furthermore, LTi cells may be important for splenic organization, since adult $CD4^+CD3^-$ cells transferred into $LT\alpha^{-/-}$ mice were able to segregate lymphocytes into B and T cell areas, which is usually impaired in these mice. Due to their tight association with $VCAM1^+$ stromal cells also in the spleen, they are in the position to elicit the secretion of homeostatic chemokines from stromal populations [252]. At last, Ludewig and coworkers showed that LTi cell – stromal cell crosstalk continues into adulthood. Acute infection with lymphocytic choriomenigitis virus (LCMV) leads to disruption of lymphoid organ integrity, with the consequence that the host becomes unable to respond to pathogens and loses immunocompetence. Mainly the $gp38^+$ fibroblastic reticular cell (FRC) network in the white pulp is affected by this disruption. After clearance of the virus however, accumulation of LTi cells in the spleen and LNs occurred with following re-establishment of the FRC network. In the absence of $CD45^+CD4^+IL\text{-}7R\alpha^+lin^-$ cells, the reorganization of the spleen was delayed [254].

Adult LTi cells also exist in humans. Cupedo *et al.* was able to detect them in postnatal tonsils [225].

NKp46+ Lymphoid Tissue inducer Cells

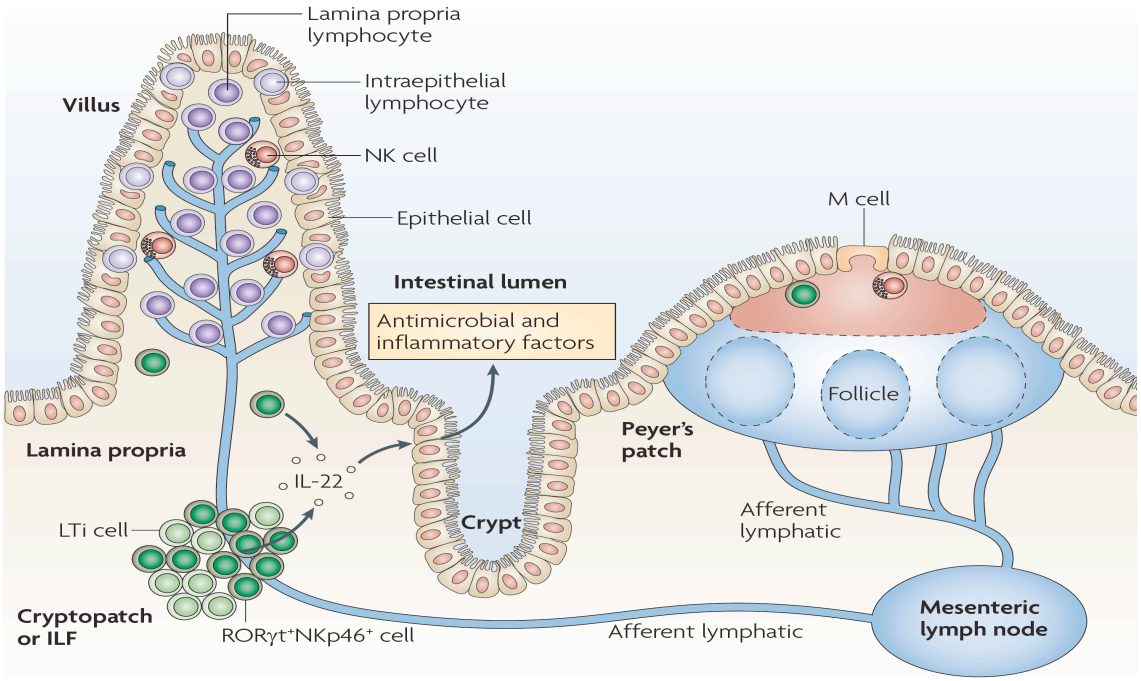

Image 9 Localization of gut RORyt+NKp46+ cells [255]

Recent studies identified an IL-22 producing population in the gut of mice, which shares features of both LTi cells and NK cells and is thought to be involved in mucosal immunity (reviewed in Vivier et al. 2009). Independent reports described this novel population in cryptopatches (CP) of the small intestine in mice [256] [257] [258] [259]. Just like LTi cells they express IL-7Rα and are positive for RORγt. However in addition they express NKp46 (also known as NCR1). NKp46 is an immunoglobulin-like transmembrane glycoprotein belonging to the family of natural cytotoxicity receptors (NCRs) and is involved in the recognition of tumor-cell targets by NK cells [260]. NKp46 is therefore usually though to be a selective marker for both mouse and human NK cells [261]. Nevertheless, there are non-NK cell populations described to express NKp46, like a discrete subset of γδT cells in mice [261] and intraepithelial lymphocyte-subsets in coeliac disease patients [262]. Unlike conventional NK (cNK) cells, RORγt$^+$NKp46$^+$IL7Rα$^+$ cells seem to develop independent of IL-15 and lack markers of mature NK cells. Further they don't posses cytotoxic effector functions as perforin and granzyme and have low or absent IFNγ production. Instead, they have been shown to be strong producers of IL-22, in particular in response to IL-23 [257] [258]. IL-22 is a member of the family of IL-10 related cytokines. It's receptor is exclusively found on epithelial cells and leads when activated to the production of antimicrobial molecules [263] [264] [265] and the anti-inflammatory cytokine IL-10 [266]. In mice, deficiency of IL-22 leads to

compromised epithelial cell barrier function in the gut und subsequent spread of bacteria [265]. Indeed, *RAG2*$^{-/-}$*IL-2Rγ*$^{-/-}$ mice which lack NKp46^{+}IL7Rα^{+}NK1.1^{-} cells, showed reduced steady-state IL-22 in the intestine that correlated with heightened susceptibility to Citrobacter rodentium [257]. Clearly, this IL-22 producing cell population contributes to the so far unknown source of IL-22 production in the gut. Splenic LTi-like cells have been shown to produce in addition to IL-22 also IL-17 [267] which has been known to induce the production of granulopoietic factors (GM-CSF and stem cell factor (SCF)). Newest findings show that this just recently identified cell type accumulates in the inflamed colon of Helicobacter hepaticus infected mice. Depletion of this population during the course of infection resulted in abrogation of both colitis and typhlitis. Buonocore *et al.* suggested that they directly mediate IL-23-dependent acute and chronic innate immune-mediated colitis through the production of inflammatory cytokines such as IL-17 and IFNγ in response to microbes [268].

Table 2 Expression profiles of adult NK cells, LTI cells and mucosal RORγt^{+}NKp46^{+} cells [255]

Protein	Human				Mouse			
	Blood NK cells	Lymph node NK cells	LTi cells	Mucosal RORγt^{+} NKp46^{+} cells	Spleen NK cells	Lymph node NK cells	LTi cells	Mucosal RORγt^{+} NKp46^{+} cells
NKp46 (CD335)	+	+	–	+	+	+	–	+
NKp44 (CD336)	+	+	–	+	NA	NA	NA	NA
NKG2D (CD314)	+	+	–	+	+	+	–	+
IFNγ	+	+	–	–	+	+	–	–
CD56 (human) NK1.1 (mouse)	+	+	–	+	+	+	–	–
KIR (human) Ly49 (mouse)	+	–	–	–	+	–	–	–
Perforin	+	–	–	–	+	–	–	–
KIT (CD117)	–	–	+	+	–	–	+	+
IL-7Rα (CD127)	–	–	+	+	–	–	+	+
IL-22	–	–	+	+	–	–	+	+
IL-17A	–	–	+	–	–	–	+	–
RORγt	–	–	+	+	–	–	+	+

+ indicates high-level expression and – indicates low-level or no expression by adult cells, based on published reports[12,14,19,20]. NA, not applicable as not expressed in mice. IFNγ, interferon-γ; IL, interleukin; KIR, killer-cell immunoglobulin-like receptor; LTi cell, lymphoid-tissue inducer cell; NK cell, natural killer cell; RORγt, retinoic acid receptor-related orphan receptor-γt.

An equivalent to this population has also been found in human tonsils and PPs. Developmentally and functionally, these mucosal RORC^{+}CD127^{+}CD56^{+}NKp44^{+} cells resemble the RORγt^{+}NKp46^{+} population found in mice [225] [2269]. A variety of culturing conditions allowed a robust differentiation of human LTi cells into these CD127^{+}CD56^{+}NKp44^{+} cells in vitro [225]. As their mouse counterpart this population produced IL-22 especially in response to IL-23 [269] and showed only minimal expression of perforin and granzyme [225]. And like human LTi cells, this population was able to induce the expression of adhesion molecules on MSCs when co-cultured in vitro [225] [270].

Despite the fact that this newly described subset expresses NKp46, their developmental dependence on RORγt and their specific tissue location suggest that RORγt^{+}NKp46^{+} cells might represent a subpopulation (or descendants) of LTi cells rather than yet another NK cell subset. Two recent studies support this assumption in mice as well as in humans. Di Santo and co-workers found that the development of both, RORγt^{+}NKp46^{+} cells as well as LTi cells, requires IL-7 and is independent of IL-15. Over expression of IL-15 however clearly expanded cNK cells in vivo. Further they observed by in vivo cell fate mapping that cNK cells never express RORc during their development, clearly dismissing a RORγt^{+} progenitor for cNK cells [271]. Crellin *et al.* showed that human Lin-CD127^{+}RORc^{+} cells in tonsils are the precursors to CD56+CD127+RORc+NKp44+ cells. Together this two cell populations comprise a stable RORc^{+} lineage and can not be conversed into cNK cells ex vivo [270]. However, LTi cells and cNK cells themselves have been reported to be developmentally related, since mice deficient for Id2 lack both mature NK cells and LTi cells [222] [271].

Table 3 Comparison between adult NK cells, LTi cells and mucosal RORγt^{+}NKp46^{+} cells [255]

Feature	LTi cells	RORγt^{+}NKp46^{+} cells	NK cells
Development			
Requirement for commensal flora	No	Yes	No
Requirement for RORγt	Yes	Yes	No
Requirement for IL-15	No	No	Yes
Localization			
Cryptopatches	Yes	Yes	No
Lamina propria	Yes	Yes	Yes
Peyer's patches	Yes	Yes	Yes
Intestinal epithelium	No	No	Yes (a few)
Tonsil	Yes	Yes	Yes
Function			
Epithelial-cell repair	ND	Yes	No
Mucosal immunity	ND	Yes	Yes
Cytolytic activity	No	No	Yes

IL-15, interleukin-15; LTi cell, lymphoid-tissue inducer cell; ND, not determined; NK cell, natural killer cell; RORγt, retinoic acid receptor-related orphan receptor-γt.

Material and Methods

Mice: C57BL/6 mice were purchased from Janvier. *Il12rb2*$^{-/-}$, *Rag1*$^{-/-}$, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, *Rorc*^{GFP} (Rorc$^{-/-}$), Prf1$^{-/-}$, *ifng*$^{-/-}$, *ifngr*$^{-/-}$ and *Itbr*$^{-/-}$ mice were purchased from Jackson Laboratories and kept in house under spf conditions. *Il15ra*$^{-/-}$ mice were kindly provided by S. Bulfone-Paus (Department of Immunology and Cell Biology, Research Center Borstel, Borstel, Germany). RORc-cre x ROSA26-stop-EYFP mice were kindly provided by A. Diefenbach (Institute of Medical Microbiology, University Hospital Freiburg, Germany). *Il17a*$^{-/-}$ mice were obtained from Y. Iwakura (University of Tokyo, Japan) and *Il22*$^{-/-}$ mice were provided by J.C. Renauld (Ludwig Inst. Brussels, Belgium). Mice were treated according to institutional animal care and use guidelines.

Bone Marrow Chimera Generation: For the generation of bone marrow chimeras, donor mice were killed by CO_2 inhalation and bone marrow cells were isolated by flushing of femur, tibia, radius and hipbones with PBS. Bone marrows cells were then passed through a cell strainer with a pore size of 100 mm and cells were washed with PBS. Recipient mice were lethally irradiated with 1,100 rads (split dose) and were injected intravenously with 12×10^6 to 25×10^6 bone marrow cells. Engraftment took place over 8 weeks of recovery. Animal experiments were approved by the Swiss Veterinary Office (10/2006; Zurich, Switzerland).

Generation of the tumor cell lines B16–IL-12, B16–IL-23 and B16-Fc: The parental B16.F10 mouse melanoma cell line (Xenogen) was stably transfected with a pCEP4 plasmid encoding the Fc fragment of mouse immunoglobulin G3 alone or IL-12 or IL-23 fused to that Fc fragment. The selection medium contained AdvMEM, 10% (vol/vol) FCS, penicillin, streptomycin, glutamine, zeocin (0.2 mg/ml) and hygromycin (0.25 mg/ml). Expression of the IL-12–Fc and IL-23–Fc fusion proteins was confirmed by enzyme-linked immunosorbent assay with anti-L-12p40 and anti-IL-23p40 (OptEIA; Pharmingen) and RT-PCR (immunoglobulin G3 forward, 5′-ACACACAGCCTGGACGC-3′, and reverse, 5′-CATTTGAACTCCTTGCCCCT-3′).

Subcutaneous inoculation of melanoma cell line and monitoring of tumor growth: A total of 1×10^5 to 10×10^5 cells were injected subcutaneously into the lateral abdomen of the mouse. Tumor growth was monitored by measurement of two perpendicular diameters of the skin tumor by caliper three times a week for a period of 3 weeks.

Analysis of lung metastasis: Three weeks after i.v. injection of B16 or B16-IL12 cells mice were euthanized and the lungs dissected. The lungs were immediately photographed.

Depletion of NK cells: Mice were injected with tumor cells on day 0 as described and injected intraperitoneal with 200 mg anti-NK1.1 (PK136; Bio X cell) on day –1, followed by three injections per week with the same amount of antibody. Depletion was confirmed by flow cytometry with an anti-CD49b (DX5). Other mice were injected intraperitoneal with 30 ml anti-GM1 (WAKO Chemicals) on day –1, followed by injection every fifth day with the same amount of antibody. Depletion was confirmed by flow cytometry with monoclonal anti-NK1.1 (PK136).

Proliferation assay: B16, B16-IL12 or B16-Fc cells (5000 cells/well) were plated into a 96-well plate in triplicates. Medium containing 0.5 mCi/ml of [3H]thymidine was added for 4 days and the incorporation assessed with a Filtermate Collector (Applied Biosystems) and a scintillation and luminescence counter. Generation of the tumor cell lines gl261-IL12 and gl261-Fc. C57/Bl6 murine glioma (Gl261) cells were stably transfected with a pCEP4 plasmid (Invitrogen) encoding either IL-12 fused to the Fc part of mIgG3 or the Fc part alone. The selection medium contained Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% GlutaMaxx (Gibco) and 0.23 mg/ml hygromycin. The resulting cell line was called GL261-IL-12-Fc or GL261-Fc respectively. The expression of the IL-12 Fc fusion protein was confirmed by ELISA using an antibody against the mouse IL-12 subunit p40 (OptEIA; Pharmingen) and rt-PCR (IgG3fw: ACACACAGCCTGGACGC IgG3rev: CATTTGAACTCCTTGCCCCT).

Protein Extraction from tumor: Three weeks after s.c. injection of B16 or B16-IL12 cells mice were euthanized and the tumors dissected. The tumors were homogenized in the presence of lysis buffer of tissue Protein Extraction Reagent (TPER, Pierce) and a protease inhibitor cocktail (Complete Mini proteease inhibitor cocktail tablets, Roche). Tumor homogenates were then assessed for p40 by ELISA kit (OptEIA; Pharmingen).

Flow cytometry: For analysis of tumor-infiltrating lymphocytes, mice were killed by CO_2 inhalation and tumors were dissected. Tumor mass was incubated for 30 min at 37 °C in RPMI medium containing DNase and Liberase, then were homogenized and strained through a nylon filter with a pore size of 100 mm (Fisher). After centrifugation, cells were incubated with antibodies for 20 min at 4 °C and then were acquired with a FACSCanto II (BD Pharmingen) and BD FACSDiva software and analyzed with FlowJo software (TreeStar).

Histology: Tumors were isolated carefully, embedded in optimal cutting temperature compound and snap-frozen in liquid nitrogen. Cryosections 6 mm in thickness were air-dried for 90 min at 25°C and fixed for 10 min at 25 °C with acetone and were then incubated with the following primary antibodies: rat anti-CD4 and rat anti-CD8 (both provided by R. Zinkernagel), armenian hamster anti-CD11c (; Serotec), rat anti-CD11b (; BMA biomedicals), rat anti–asialo GM1 (NK cell; ;Wako Chemicals), rat anti-F4/80 (; BMA biomedicals), rat anti-Gr-1 (; PharMingen–Becton Dickinson), goat anti-Nkp46 (; R&D Systems), rabbit anti-GFP-YFP (; Abcam), rabbit anti-CD31 (; PharMingen–Becton Dickinson), rabbit anti-CD54 (ICAM; Serotec) and rabbit anti-CD106 (VCAM-1; Serotec). For conventional immunohistochemistry, antibodies were coupled to alkaline phosphatase or were detected with alkaline phosphatase–coupled secondary antibodies. Staining was visualized with Fast Red as the alkaline phosphatase substrate (Refine Detection kit; Leica). Brightfield images were acquired with a 50× (Numerical aperture 0.9) objective on a Olympus BX41 light microscope equipped with a ColorView IIIu camera (Olympus) and Cell B software (Olympus). For immunofluorescence staining, primary antibodies were detected with secondary antibodies coupled to Alexa Fluor 488 or tetramethylrhodamine isothiocyanate and sections were mounted in fluorescence mounting medium containing DAPI (4′,6-diamidine-2-phenyl indole; Vectashield; Vector). Images were acquired with 20× (N.A. 0.7) and 63× (Numerical aperture 1.4) objectives on Leica SP5 confocal laser-scanning

microscope equipped with LAS AF (Leica application suite advanced fluorescence) 2.2.1 software (Leica) and were further analyzed with Imaris 7.1.1 software (Bitplane). Maximum intensity projections are presented.

In vivo expansion of LTi cells: Rorc-EYFP mice were treated with á-IL-7/IL-7 complex as described by Schmutz et al.39. LTi cells were isolated using a high-yield pre-sort for EYFP+ followed by a low-pressure, high purity sort for EYFP+CD3-.

In vitro stimulation of LTi cells: The cells were cultured on a non-confluent monolayer of OP9-feeder cells in the presence of 10ng/ml of IL-7 with or without 2.5 ng/ml rIL-12
for 24h.

RNA Isolation and cDNA Synthesis: Cells were harvested and RNA isolated according to manufactures protocol (). For cDNA synthesis, 1-5 ìg RNA was reverse transcribed using reverse super transcriptase III (Invitrogen), which uses single stranded RNA to synthesize a complementary cDNA strand in the presence of a primer. The following components (all from Invitrogen) are mixed gently together in a tube and heated at 37°C for 5 min prior to adding M-MLV RT: 5X First strand buffer, dNTPs, DTT, random primers and Recombinant Ribonuclease Inhibitor RNaseOUT. The mix is then added to the RNA and incubated at RT for 10 min, 37°C for 50 min and 70°C for 15 min to inactivate the reaction.

Real-time PCR Analysis: Diluted cDNA was mixed with the following components. Real-time PCR was carried out to analyze RNA expression of cytokines and chemokines using the following primer sequences:

Statistical analysis: An unpaired two-tailed Student’s t-test was used to determine the statistical significance of differences. P values of less than 0.05 were considered statistically significant.

Results

The Model

B16 cell lines

To determine the mechanistic underpinnings by which IL-12 mobilizes antitumor immunity, we generated a melanoma cell lines that continuously released a fusion protein of IL-12 and the crystallizable fragment (IL-12–Fc), IL-23–Fc or mouse immunoglobulin G3–Fc alone as controls (called 'B16–IL-12', 'B16–IL-23' and 'B16' here, respectively). We confirmed secretion of IL-12 and IL-23 by enzyme-linked immunosorbent assay (**Fig. 1a and b**). The expression of the Fc-tail was confirmed by RT-PCR (**Fig. 1c**). The advantage of using fusion proteins over the recombinant cytokine is the improved pharmacokinetics [272 273 274]. The use of these modified melanoma cell lines serves the purpose to identify the cell type responsive to IL-12 rather than to determine the therapeutic value of IL-12.

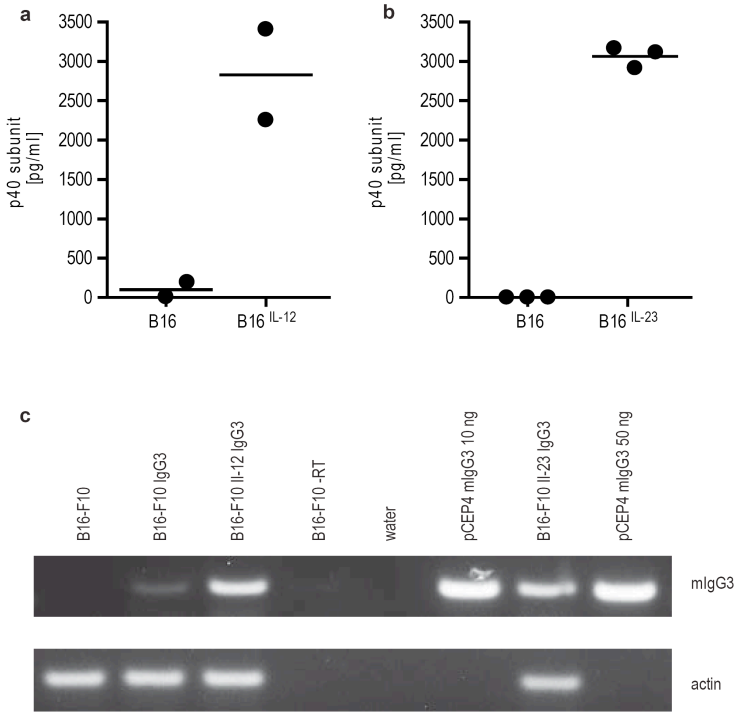

Figure 1 Confirmation of the cell lines

(**a**) and (**b**) Confirmation of IL-12/23 p40 secretion by (**a**) B16–IL-12 and (**b**) B16–IL-23 cells. Parental B16-F10 cell lines were transformed with an episomal expression vector (pCEP-Invitrogen) encoding IL-12–Fc, IL-23–Fc or IgG3–Fc alone and maintained in selective medium for four weeks. Expression of the fusion protein was confirmed by ELISA, which detects the p40 subunit of IL-12 and IL-23. Each data point represents triplicates. Data represent three experiments. (**c**) Confirmation of IgG3 by rt-PCR.

IL12 significantly induces Tumor Suppression

Subcutaneous injection of 2×10^5 B16 cells into the lateral abdomen of wild-type mice induced a single solid tumor mass, reaching a size of ~300 mm^2 after 21 days. Mice injected with 2×10^5 B16–IL-12 cells had much smaller tumor masses than did those injected with the parental B16.F10 cell line or B16-Fc cells (**Fig. 2a**). Wild-type mice injected with control B16 cells secreting IL-23 instead of IL-12, however, did not suppress tumor growth (**Fig. 2b**). We confirmed IL-12 secretion in the tumor by enzyme-linked immunosorbent assay (**Fig. 2c**).

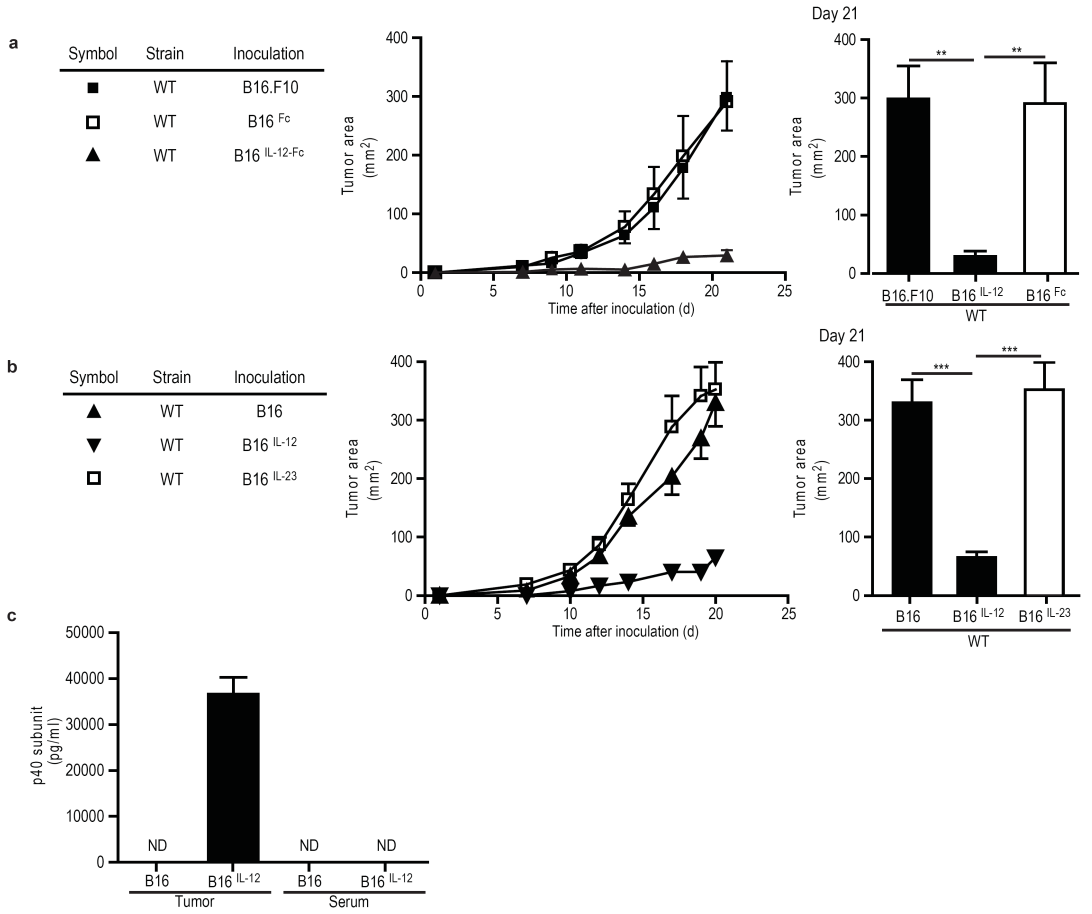

Figure 2 IL-12 significantly induces tumor suppression

(**a**) Repression of subcutaneous tumors in wild-type mice given subcutaneous injection of 2×10^5 B16.F10, B16–IL-12 or B16-Fc cells (n = 6 mice per group). Data are representative of four experiments. (**b**) Tumor suppression in wild-type mice given subcutaneous injection of $2x10^5$ B16, B16–IL-12 or B16–IL-23 cells (n = 6 mice per group). Data are representative of three experiments. (**c**) IL-12-p40 secretion in the tumor vs. serum in wild-type mice given subcutaneous injection of $2x10^5$ B16 or B16–IL-12 cells (n = 6 mice per group). Tumors and sera were harvested 21 days post injection. Expression of the fusion protein was confirmed by anti p40 ELISA. Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test).

Long-term tumor monitoring showed that the secreted IL-12 significantly ($p<0.001$) slowed tumor growth in wild-type mice (**Fig. 3a**). The ability of IL-12 to mediate tumor repression was not restricted to the melanoma model B16.F10. Subcutaneous injection of 2×10^6 GL261 cells, a syngeneic glioma cell line, expressing either IL-12–Fc or Fc alone into *Rag1*$^{-/-}$ mice resulted in similar tumor suppression (**Fig. 3b**). IL-12-mediated tumor suppression was not restricted to IL-12 secretion by transfected B16 cells but was also induced by systemic intra peritoneal (i.p.) application of recombinant IL-12 ^{189} (**Fig. 3c**).

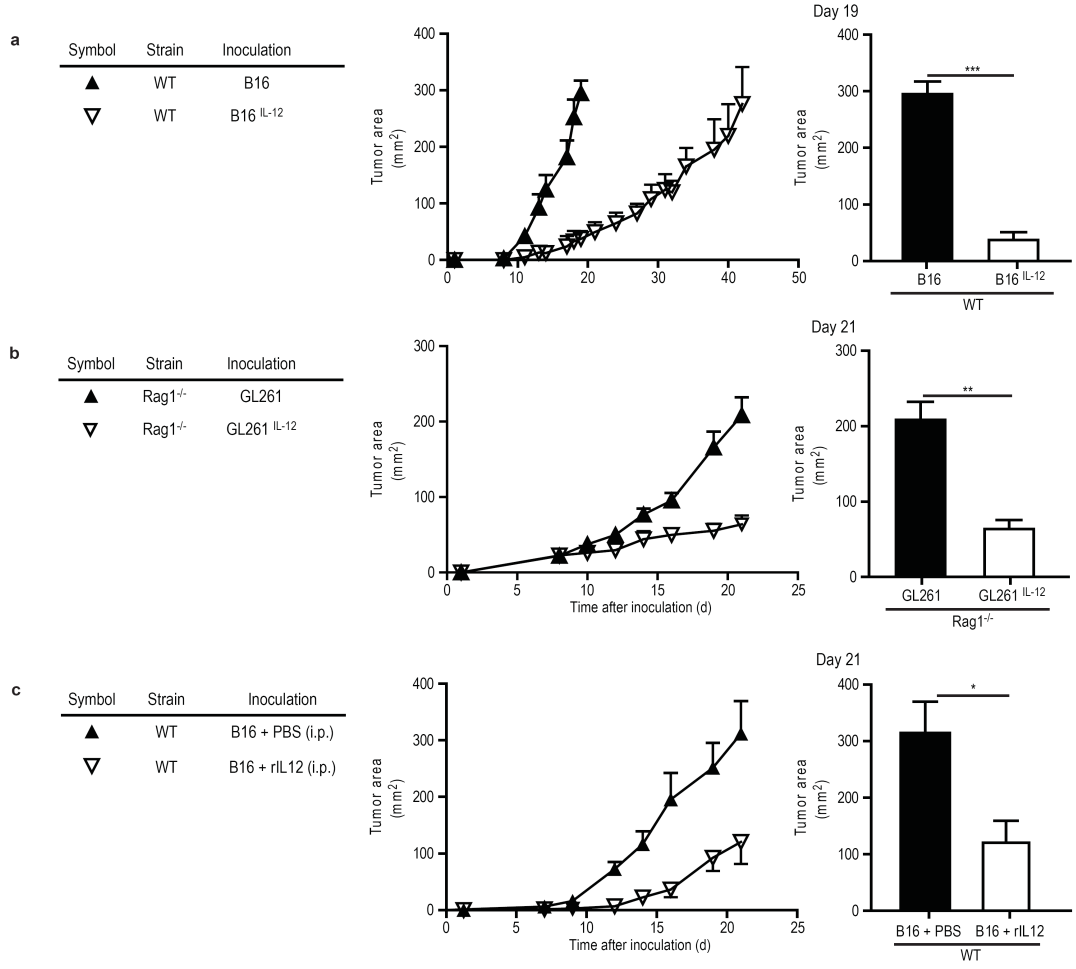

Figure 3 IL-12 mediated suppression (long-term / GL261 / intraperitoneally)

(**a**) Long-term repression of subcutaneous tumors in wild-type mice given subcutaneous injection of 2×10^5 B16–IL-12 or B16-Fc cells (n = 6 mice per group). Tumor growth was monitored for 42 days. Data are representative of three experiments. (**b**) Repression of subcutaneous tumors in *Rag1*$^{-/-}$ mice given subcutaneous injection of $2x10^6$ GL261–IL-12 or GL261–Fc cells mice (*n* = 6 mice/group). Data are representative of two experiments. (**c**) Tumor repression in wild-type mice given subcutaneous injection of 2×10^5 B16 cells and treated intraperitoneally (i.p.) with PBS or recombinant IL-12 (rIL-12) on day 1, 3, 5 and 9 after tumor injection (*n* = 3 mice per group). Shown are the mean and s.e.m.; NS, not significant. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (Student's *t*-test).

Paracrine fashion of IL-12

IL-12 could have been affecting tumor cell proliferation to mediate tumor suppression. However, B16–IL-12 and B16-Fc cells proliferated *in vitro* approximately at the same rate as the parental cell line did (**Fig. 4a**). Thus, anti-hyperplasia cannot explain the repressive effect of IL-12 on B16 tumor growth. To investigate whether IL-12 functioned in a paracrine way on host cells, we mixed 1×10^5 B16 cells with B16–IL-12 cells at a ratio of 1:1 and subcutaneously injected the mixture into wild-type mice. Tumor development was diminished to an extent similar to that when B16–IL-12 cells were injected alone. Even an increase in the ratio in favor of B16 cells (1:2 and 1:10) did not extinguish the strong tumor-repressive effect of the co injected B16–IL-12 cells (**Fig. 4b**), which indicated that the secreted IL-12 acted in a paracrine way. That idea was further supported by the finding that B16–IL-12 cells showed uninhibited growth in *Il12rb2*$^{-/-}$ mice, which lack the IL-12-specific receptor subunit (**Fig 4c**).

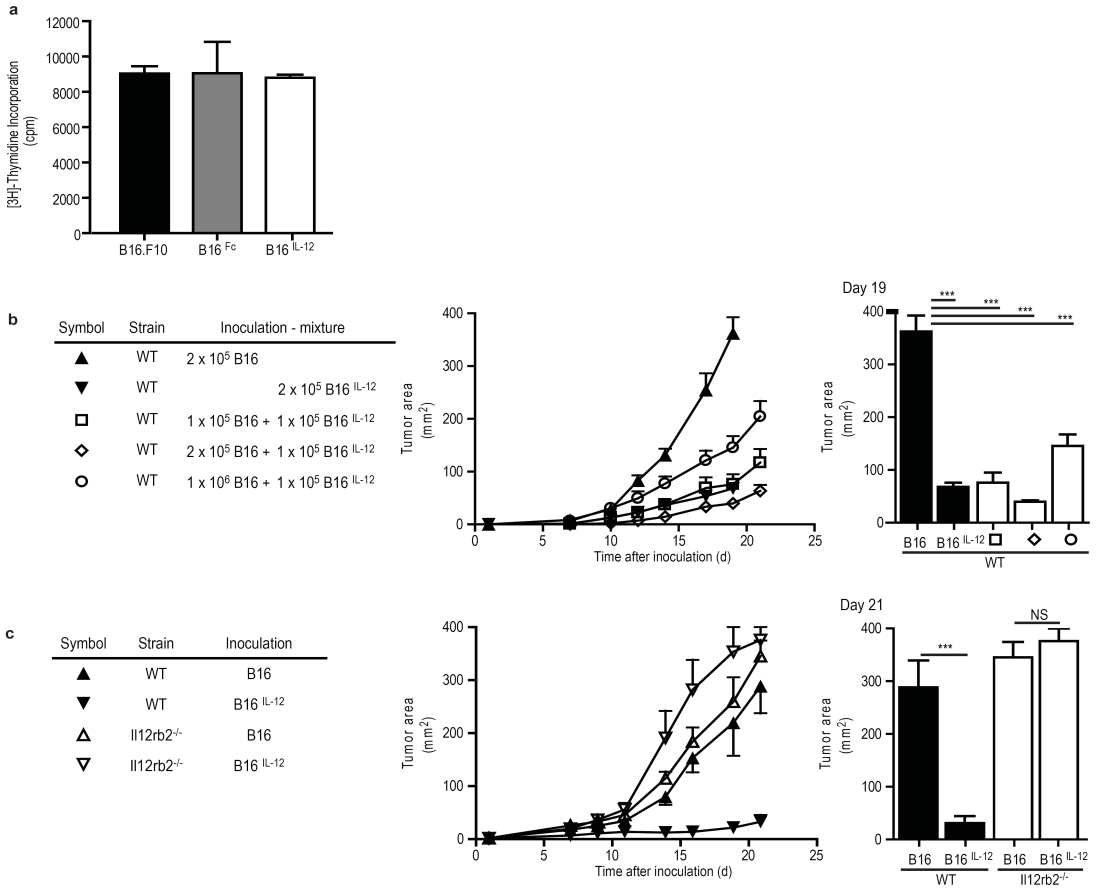

Figure 4 IL-12 acts in a paracrine way

(**a**) In vitro proliferation of B16.F10, B16–Fc B16–IL-12. Cells were cultured in 96-well plates (triplicate-wells) and the incorporation of 3[H]thymidine monitored after 4 days. Data are representative of three experiments. (**b**) Repression of tumors in wild-type mice given subcutaneous injection of a mixture of B16 cells and B16–IL-12 cells at a ratio of 1:1, 2:1 or 10:1 (n = 5 mice per group). Data are representative of at least two experiments. (**c**) Repression of tumors in wild-type or *Il12rb2*$^{-/-}$ mice given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells (n = 6 mice per group). Data are representative of three experiments. Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

Local versus Systemic Effect of IL-12

To further distinguish local versus systemic effects of secreted IL-12, we subcutaneously injected 2×10^5 B16 cells into the right lateral abdomen and 2×10^5 B16–IL-12 cells into the left lateral abdomen of wild-type mice. After 3 weeks, B16–IL-12 tumors showed only minimal growth, whereas B16 tumors on the right flank grew normally and were unaffected by the contra lateral B16–IL-12 cells (**Fig. 5a**). This demonstrates that IL-12 secretion acts locally and cannot repress the growth of tumors implanted at a distant site. The micro environmental effect of IL-12 suggested that it acts through the innate immune system rather than involving lymphocytes. Even injection of recombinant IL-12 into an established palpable tumor 7 d after tumor transfer significantly suppressed tumor growth (**Fig. 5b**).

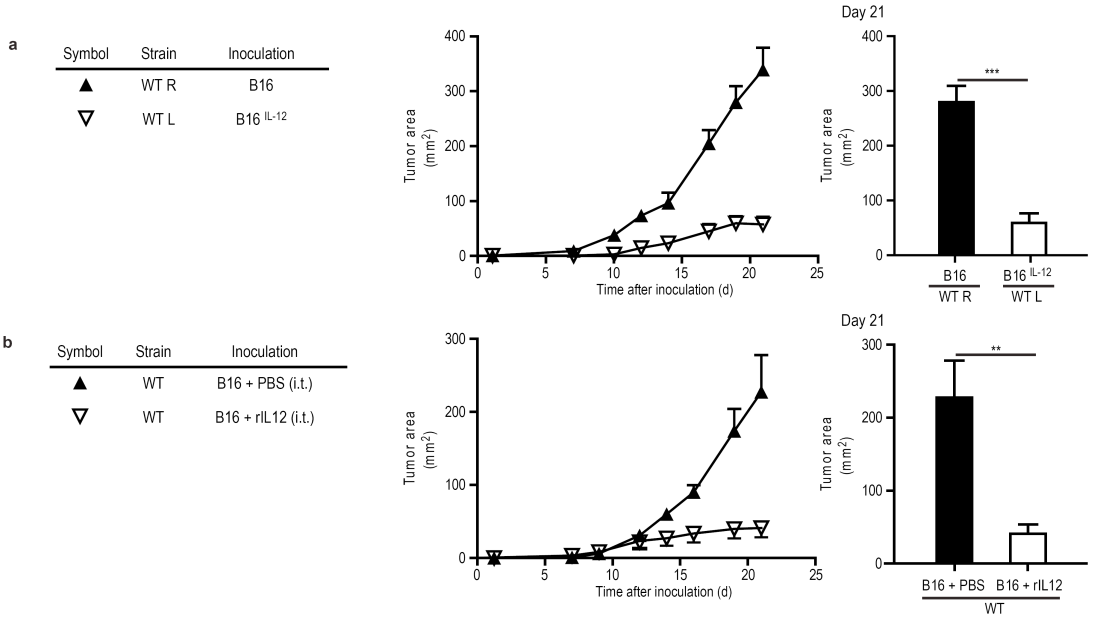

Figure 5 IL-12 acts locally

(**a**) Tumor repression in wild-type mice given subcutaneous injection of 2×10^5 B16 cells in the left abdomen and 2×10^5 B16–IL-12 cells in the right lateral abdomen (n = 6 mice per group). Data are representative of three experiments. (**b**) Tumor repression in wild-type mice given subcutaneous injection of 2×10^5 B16 cells and intratumoral (i.t.) injection of PBS or recombinant IL-12 on days 7, 9, 12, 14, 16 and 19 after tumor injection (n = 3 mice per group). Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

IL-12 Effect in Lung Metastasis

Because B16–IL-12 cells had such a strong repressor phenotype on subcutaneously tumor growth, we also investigated the effect of IL-12 on tumor metastasis. We injected 1×10^5 B16 or B16–IL-12 cells intravenously into wild-type mice and, at 21 days after injection, we analyzed metastasis formation in the lungs. Although B16 cells formed many metastases, mice injected with B16–IL-12 cells did not develop lung metastasis (**Fig. 6a and b**), which indicated that IL-12 suppresses not only subcutaneous primary melanoma but also the formation of metastasis.

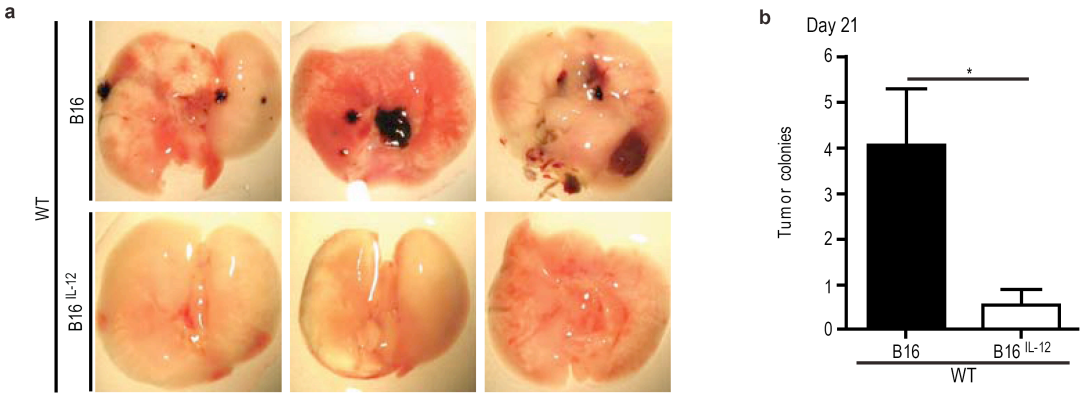

Figure 6 IL-12 mediated repression of lung metastasis

(**a**) and (**b**) Repression of lung tumors in wild-type mice given intravenous injection 1x105 B16 (upper row) or B16-IL-12 (lower row) ($n \geq 4$ mice per group). Mice were sacrificed after 21 days and lungs were analyzed (**a**) and counted (**b**) for the formation of metastasis. Data are representative of two experiments. Shown are the mean and s.e.m.; NS, not significant. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test).

IL-12 acts independent of Lymphocytes, NK cells or Myeloid cells

CD45 Infiltration

Histological analysis 3 weeks after inoculation showed that B16–IL-12 cells recruited more leukocytes to the tumor site than did B16 cells (**Fig. 7a**). Flow cytometry of the tumor 3 weeks after inoculation showed that in B16 tumors, $CD45^+$ leukocytes represented only <10% of the total cells, whereas in B16–IL-12 tumors, >30% were $CD45^+$ leukocytic infiltrates (**Fig. 7b**). There were significantly more $CD8^+$ and $CD4^+$ cells as well as $CD11c^+$ and $NK1.1^+$ cells in B16–IL-12 tumors than in B16 tumors, whereas there were fewer infiltrating $CD11b^+$ cells and neutrophils (Ly6G, clone $1A8^+$) (**Fig. 7c - i**).

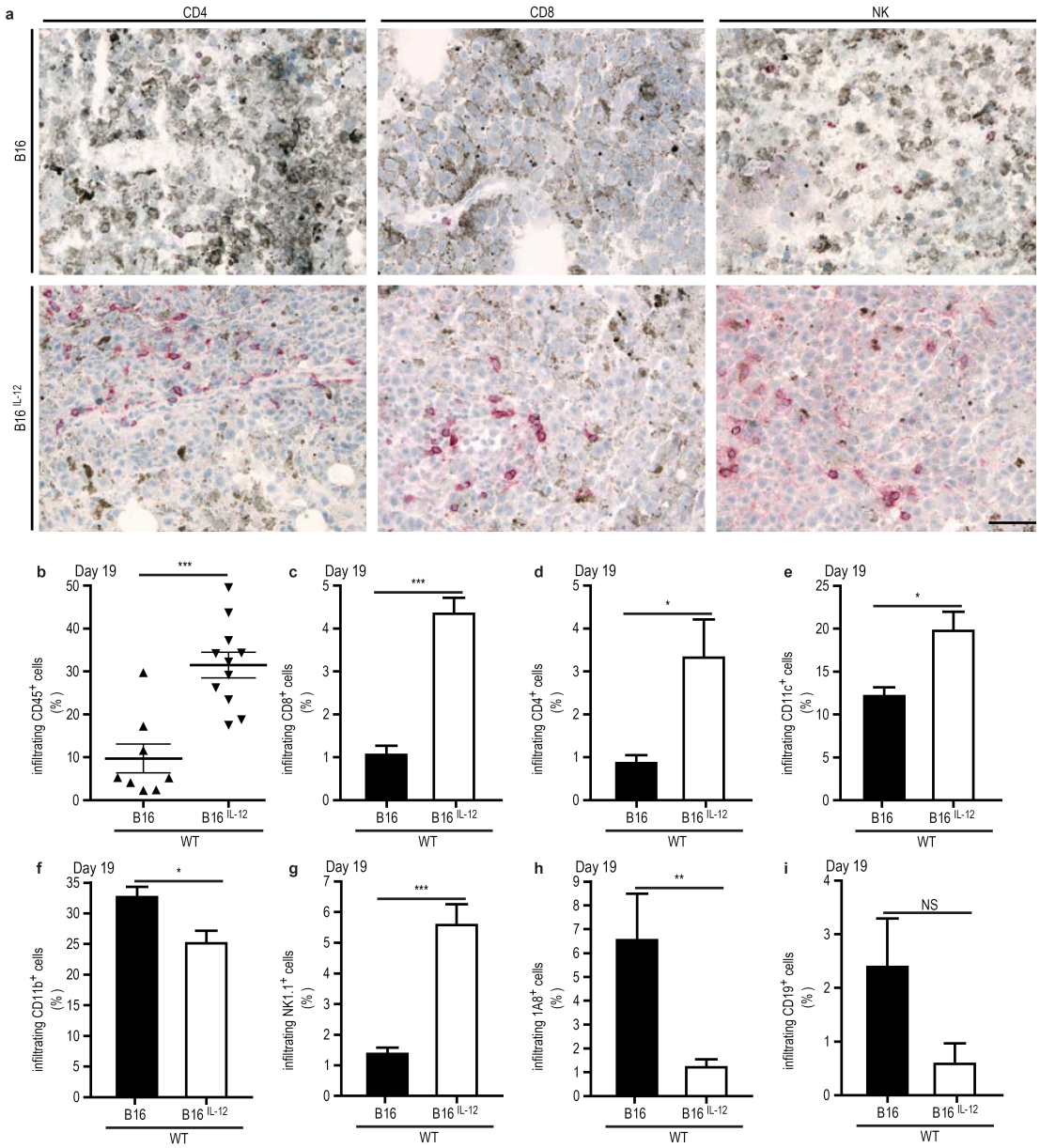

Figure 7 IL-12 elicits the recruitment of leukocytes into the tumor mass

(**a**) Immunohistochemistry of frozen tumor sections obtained from wild-type mice 3 weeks after challenge with B16 or B16–IL-12 cells and stained with anti-CD4 (CD4), anti-CD8 (CD8) and anti–asialo GM1 (NK). Scale bar, 50 μm. (**b**) Cytofluorometry of tumor-invading leukocytes in mice treated as in **a**, assessed in the entire tumor mass after exclusion of cellular debris, dead cells and duplets, and presented as tumor-infiltrating $CD45^+$ leukocytes relative to $CD45^-$ melanoma cells. Each symbol represents an individual mouse; large horizontal lines indicate the mean and short lines the s.e.m.. (**c**–**i**) Cytofluorometry of tumor-invading leukocytes in mice treated as in **a**, presented as the frequency of infiltrating $CD8^+$ cells (**c**), $CD4^+$ cells (**d**), $CD11c^+$ cells (**e**), $CD11b^+$ (**f**), $NK1.1^+$ cells (**g**), $1\text{-}A8^+$ cells (**h**) and $B220^+$ cells (**i**), gated on $CD45^+$ cells, in B16 tumors relative to that in B16–IL-12 tumors. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*-test). Data are representative of two experiments with at least four mice per group (mean and s.e.m.).

Stroma versus Immune System

In order to discern whether these tumor-invading leukocytes or IL-12-responsive tumor-surrounding stroma cells initiate tumor suppression, we generated a series of bone-marrow (BM) chimeric mice. BM-chimerism allows separating the genotype of the hematopoietic system from the radio-resistant stroma after lethal irradiation of the mouse and the reconstitution by hematopoietic stem cells [275] [276] [277]. For our purpose, we used irradiated wt mice and reconstituted them with IL-12Rb2$^{-/-}$ stem cells and vice versa. After engraftment, the mice were challenged with 2x10^5 B16 and B16–IL-12 respectively. IL-12 was able to induce tumor suppression in *Il12rb2*$^{-/-}$ mice reconstituted with wt bone marrow expressing the IL-12 receptor only in the hematopoietic compartment. On the other hand, when the IL-12Rβ2 expression is restricted to the radio-resistant stromal compartment, the injected B16–IL-12 tumors showed the same tumor growth as the B16 cells (data not shown). This data suggests that secretion of IL-12 by the tumor activates a component of the immune system rather than a cell population belonging to the stroma.

T / NKT cells

IL-12 is known to play a predominant role in promoting T_H1 responses; also T cells represent the dominant tumor-infiltrating cell type. In order to investigate the role of T cells in IL-12 mediated tumor repression, we analyzed the growth of B16–IL-12 in *Rag1*$^{-/-}$ mice. In agreement with Kodama et al., B16–IL-12 tumor growth continued to be repressed even in the absence of B, T and NKT cells excluding their involvement in the IL-12-mediated repression of subcutaneous tumors (**Fig. 8a**) [185].

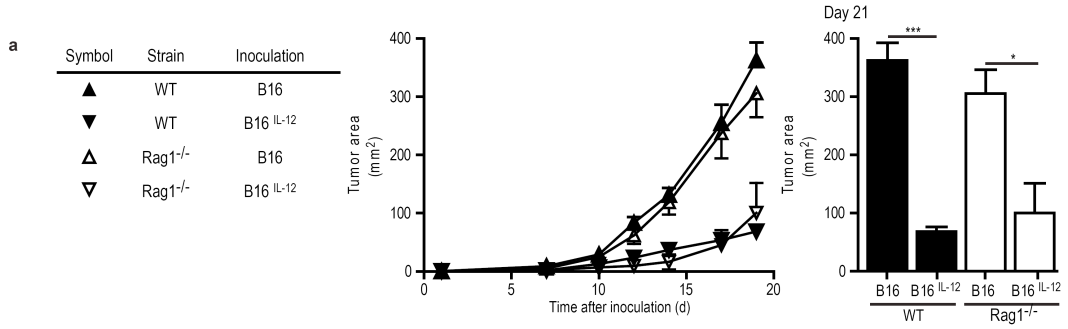

Figure 8 T cells, B cells and NKT cells independent IL-12-mediated repression of subcutaneous tumor

(**a**) Tumor repression in wild-type and *Rag1*$^{-/-}$ mice given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells ($n \geq 6$ mice per group). Data are representative of four experiments. Shown are the mean and s.e.m.; NS, not significant. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (Student's *t*-test).

conventional NK cells

Apart from T cells, another potential candidate effector population, known to be activated by IL-12 and to have tumoricidal activity, is conventional NK (cNK) cells, which express the cell surface protein NK1.1. Therefore, we depleted cNK cells from *Rag1*$^{-/-}$ mice by repeated administration of a depleting αNK1.1 antibody (PK136). Ablation of these cNK cells in the spleen and in the tumor itself was confirmed by flow cytometric analysis using anti-DX5 (**Fig. 9a and b**). However, B16-IL-12 melanoma cells were still well controlled in NK-depleted *Rag1*$^{-/-}$ mice, indicating that cNK cells are not involved in IL-12-mediated repression of subcutaneous tumors (**Fig. 9c**). The same was true for *Rag1*$^{-/-}$ mice depleted of cNK by repeated administration of antibody to the glycolipid asialo GM1 (**Fig. 10a and b**). Ablation of these cNK cells in the spleen and in the tumor itself was confirmed by flow cytometric analysis using anti-NK1.1. Again, B16-IL-12 melanoma cells were still well controlled (**Fig. 10c**).

One could however argue that the depletion was not 100 percent complete and that surviving cNK cells mediated the effect. To verify that cNK cells are not the IL-12-driven tumor controlling entities, we took advantage of the dependence of cNK cell development on IL-15 [278]. The IL-15Rα is a high-affinity binding receptor for IL-15 and mice lacking IL-15 or IL-15Rα are completely deficient in cNK cells [279]. Confirming that IL-12 does not cause tumor suppression through the activity of cNK cells in vivo, B16-IL-12 tumor cells were successfully controlled in *Il15ra*$^{-/-}$ mice (**Fig. 11**).

Notably, the effect of IL-12 on metastasis formation in the lung was abrogated by the application of anti-NK1.1 in *Rag1*$^{-/-}$ mice intravenously inoculated with 1×10^5 B16–IL-12 cells (**Fig. 12a and b**), which indicated that IL-12-mediated tumor suppression in lung metastasis differed from the suppression of subcutaneous tumors [280] [189].

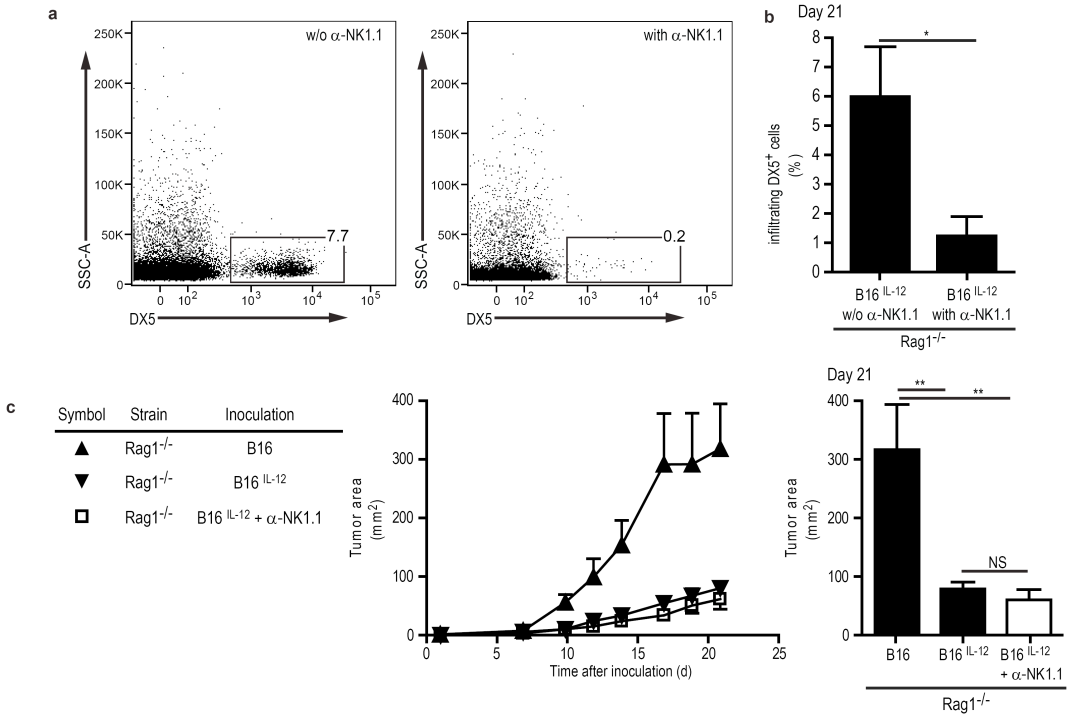

Figure 9 Anti-NK1.1 - mediated NK cell depletion in subcutaneous tumors

(**a**) B16 tumors from *Rag1*-/- (left dot plot) and anti-NK1.1 treated *Rag1*-/- (right dot plot) mice were analyzed by flow cytometry for the depletion of NK cells with DX5 antibody three weeks after inoculation. (**b**) Pooled data from n = 5 mice per group. Data are representative of two experiments. (**c**) Tumor repression in $Rag1^{-/-}$ and anti-NK1.1 (α-NK1.1)-treated $Rag1^{-/-}$ mice given subcutaneous injection of 2×10^5 B16–IL-12 cells ($n \geq 3$ mice per group). Data are representative of two experiments. Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's t-test).

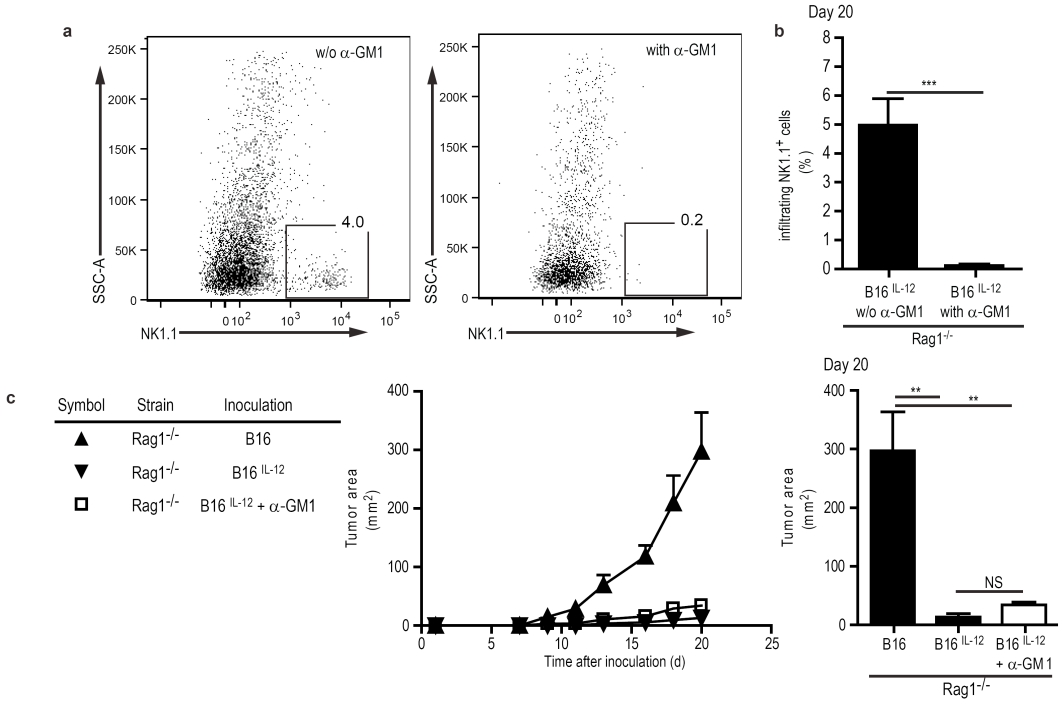

Figure 10 Anti-GM1 - mediated NK cell depletion in subcutaneous tumors

(**a**) B16 tumors from *Rag1*-/- (left dot plot) and anti-GM1 treated *Rag1*-/- (right dot plot) mice were analyzed by flow cytometry for the depletion of NK cells with NK1.1 antibody three weeks after inoculation. (**b**) Pooled data from $n = 5$ mice per group. (**c**) Tumor repression in $Rag1^{-/-}$ and anti-GM1 (α-GM1)-treated $Rag1^{-/-}$ mice given subcutaneous injection of 2×10^5 B16–IL-12 cells ($n = 5$ mice per group). Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test).

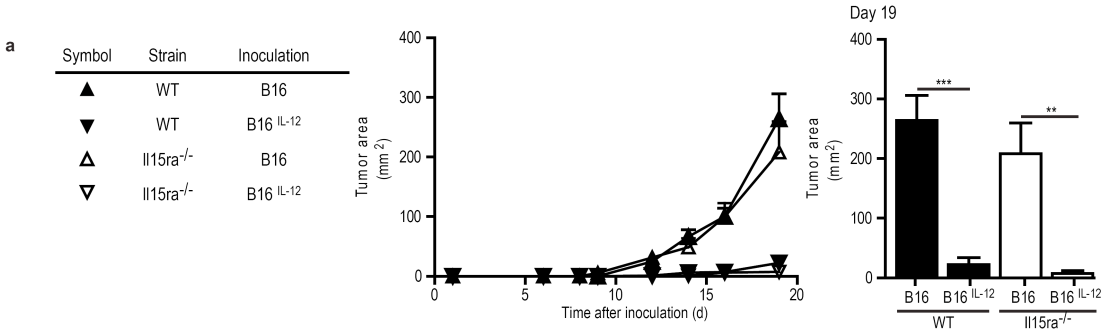

Figure 11 IL-12-mediated repression acts independent of IL-15

(**a**) Tumor repression in wild-type and *Il15ra*$^{-/-}$ mice given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells ($n \geq 6$ mice per group). Data are representative of two experiments. Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test).

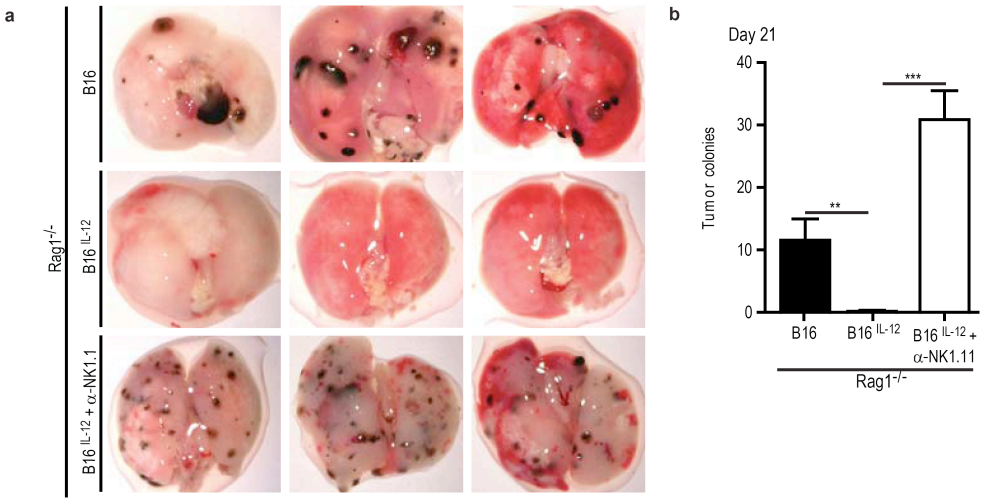

Figure 12 IL-12 mediated repression fails in NK-depleted lung metastasis

(**a**) Lung metastasis in *Rag1*$^{-/-}$ mice given intravenous injection of 1×10^5 B16 or B16–IL-12 cells ($n = 6$ mice per group), followed by depletion of NK cells with anti-NK1.1 (three times per week). (**b**) Metastases were counted after 21 days. Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test).

Macrophages and Dendritic cells

To identify the IL-12-responsive cell type responsible for tumor suppression, we took advantage of the unrestricted growth of B16–IL-12 tumor cells in *Il12rb2*$^{-/-}$ mice. We simultaneously injected *Il12rb2*$^{-/-}$ mice with a 1:1 mixture of 2×10^5 B16–IL-12 cells and splenocytes obtained from wild-type, *Rag1*$^{-/-}$ or *Il12rb2*$^{-/-}$ mice. Co-injection of B16–IL-12 cells and IL-12R-deficient splenocytes did not retard tumor growth whereas co-injection of B16–IL-12 cells and wild-type or Rag1-deficient splenocytes suppressed tumor growth (**Fig. 13**). This experimental setup allowed us to assess the capacity of fractionated cell populations to control the tumor.

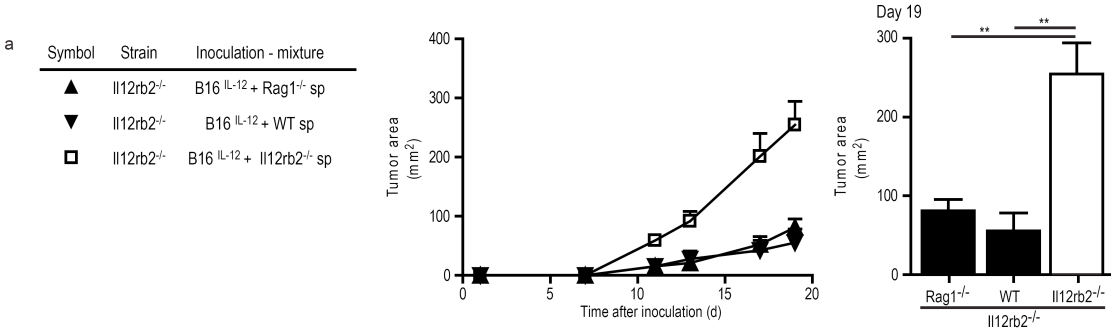

Figure 13 Adoptive transfer of leukocytes bearing IL-12R reestablishes tumor suppression in *Il12rb2*$^{-/-}$ mice

(**a**) Tumor repression in *Il12rb2*$^{-/-}$ mice given subcutaneous co injection of 2×10^5 B16–IL-12 cells and 2×10^5 splenocytes from wild-type, *Rag1*$^{-/-}$ or *Il12rb2*$^{-/-}$ mice ($n \geq 5$ mice per group). Data are representative of at least three experiments. Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test).

Not only T cells and cNK cells express the IL-12 receptor complex, it is also present on macrophages and dendritic cells (DCs) [281]. In addition, it has been shown that so-called tumor-associated macrophages respond to changes in the microenvironment surrounding tumors [39]. In particular, myeloid suppressor cells have been shown to influence the tumor microenvironment and can provide a tumor-suppressing or supporting milieu [37]. Further, Wesa *et al.* recently demonstrated that a subset of DCs has tumoricidal activity [61]. Therefore macrophages and DCs were possible candidates to be directly involved in IL-12-induced tumor control. To investigate their impact in our model, CD11b$^+$ and CD11c$^+$ cells respectively were fractionated by cell sorting from RAG1-deficient splenocytes and co-injected into *Il12rb2*$^{-/-}$ mice together with 2x10^5 B16–IL-12 cells. We found that neither sorted CD11b$^+$ (macrophages) nor CD11c$^+$ (DCs) (data not shown) cells had any impact on the B16–IL-12 phenotype in *Il12rb2*$^{-/-}$ mice.

IL-12 represses subcutaneous Tumor Growth via NKp46+ LTi cells

The troubling data about the *Rag2$^{-/-}$Il2rg$^{-/-}$* mice

Although our data suggested IL-12-mediated tumor suppression did not involve cNK cells, a published study and our results have demonstrated that in mice lacking genes encoding the RAG recombinase as well as the common γ-chain (*Il2rg$^{-/-}$*), IL-12 failed to repress tumor growth [189] (**Fig. 14a**). We also found that injection of unfractionated leukocytes from *Rag2$^{-/-}$Il2rg$^{-/-}$* mice together with B16–IL-12 cells failed to suppress tumor growth compared with cells from *Rag1$^{-/-}$* or wild-type mice (**Fig. 14b**). This showed that in contrast to *Rag1$^{-/-}$* mice, *Rag2$^{-/-}$Il2rg$^{-/-}$* mice lacked the IL-12-responsive tumor-suppressor cell type. The authors of the previously published study concluded that IL-12 activates a population of cytotoxic dendritic cells is responsive to IL-12 and kills tumor cells [189]. However, in addition to lacking cNK cells, *Rag2$^{-/-}$Il2rg$^{-/-}$* mice also lack LTi cells, because of defective signaling through the IL-7 receptor [253].

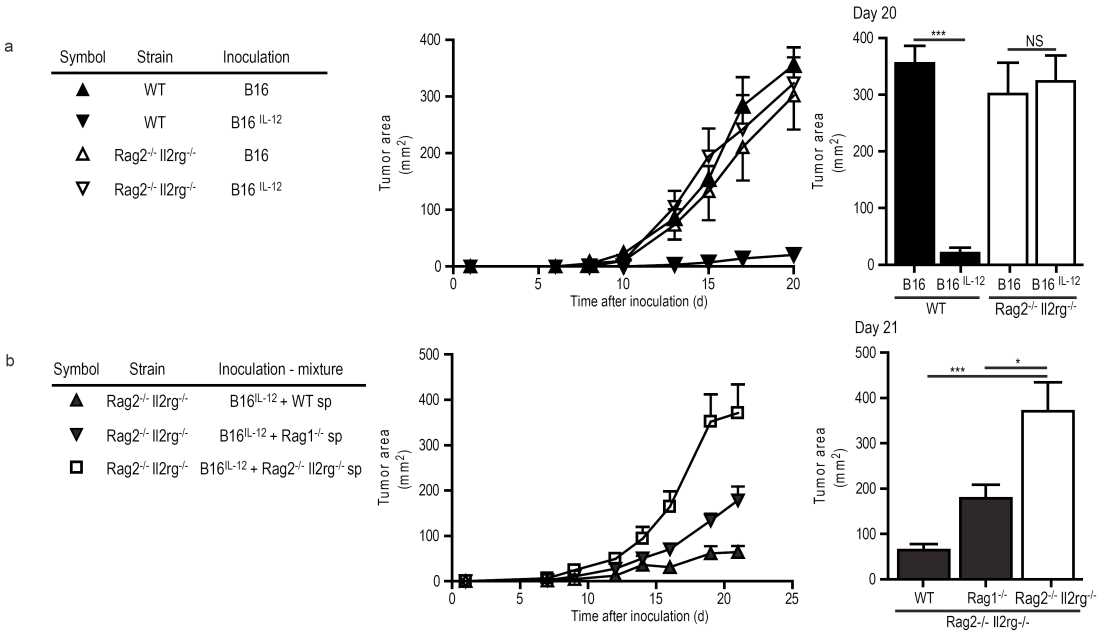

Figure 14 IL-12-mediated repression is dependent on the common γ-chain

(**a**) Tumor suppression in wild-type and *Rag2$^{-/-}$Il2rg$^{-/-}$* mice given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells ($n \geq 6$ mice per group). Data are representative of four experiments. (**b**) Tumor suppression in *Il12rb2$^{-/-}$* mice given subcutaneous co injection of 2×10^5 B16–IL-12 cells and 2×10^5 splenocytes from wild-type, *Rag1$^{-/-}$* or *Rag2$^{-/-}$Il2rg$^{-/-}$* mice ($n \geq 6$ mice per group). Data are representative of three experiments. Shown are the mean and s.e.m.; NS, not significant. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test).

NKp46+ LTi cells

Another cell type functionally related to LTi cells has been identified [23–26]. This LTi subtype has some phenotypic features in common with cNK cells (expression of the putative viral hemaglutinin receptor NKp46) but shows low to no expression of NK1.1 and CD49b. NKp46, an immunoglobulin-like transmembrane glycoprotein, belongs to the NCR family involved in the recognition of tumor targets [33]. The idea that this NKp46$^+$ LTI cell is the IL-12-responsive tumor suppressor would tally with the conflicting findings regarding the phenotype of the *Il15ra*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. To determine whether this cell type invades the tumor in an IL-12-dependent fashion, we analyzed the expression of NKp46 by immunohistochemistry. We observed large numbers of NKp46$^+$ cells in IL-12 secreting tumors (**Fig. 15**, lower left panel) while in B16 tumors (**Fig. 15**, upper left panel), NKp46 was virtually absent. As already mentioned, the NKp46 receptor is also expressed by cNK cells, and most of the detected NKp46$^+$ cells within the B16–IL-12 tumor seem to be cNK cells as shown earlier (**Fig. 7a**). As expected, in the *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ tumors (B16 and B16-IL-12) anti-NKp46 staining was absent (**Fig. 15**, middle panels). To determine whether cNK cells or NKp46$^+$ LTi cells are recruited into the tumor mass, we transplanted B16 and B16-IL-12 cells into cNK-deficient *Il15ra*$^{-/-}$ mice. (**Fig. 15**, lower right panel) clearly shows the presence of NKp46$^+$ cells, which do not belong to the cNK cell pool indicating that LTi cells mediate IL-12-driven tumor suppression.

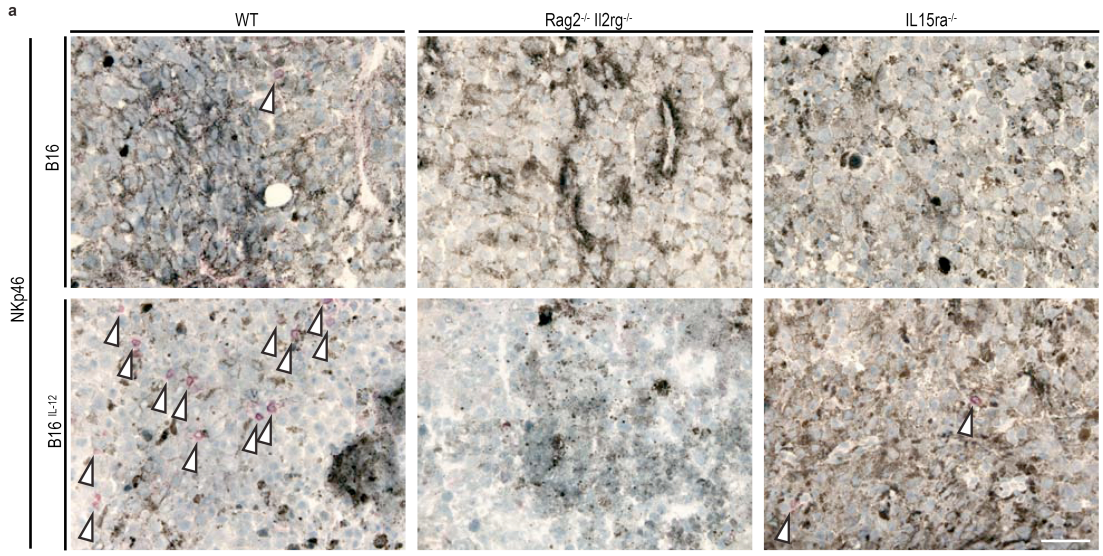

Figure 15 IL-12 induces the invasion of NKp46$^+$ cells into the tumor mass

(**a**) Immunohistochemistry of frozen tumor sections obtained from wild-type, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and *Il15ra*$^{-/-}$ mice 3 weeks after challenge with B16 or B16–IL-12 cells and stained with anti-NKp46 ($n \geq 6$ mice per group). Arrowheads indicate NKp46$^+$ cells. Scale bar, 50 μm.

To solidify a functional impact of this cell type in tumor suppression *in vivo,* NKp46^{+} LTi cells were sorted by flow cytometry for NKp46^{+}NK1.1/DX5^{low} from *Rag1*$^{-/-}$ and *Il12rb2*$^{-/-}$ splenocytes respectively and co-injected with 2x10^{5} B16–IL-12 cells into *Il12rb2*$^{-/-}$ mice. Strikingly, the co-implantation of only 6x10^{3} *Rag1*$^{-/-}$ NKp46^{+}NK1.1^{low}DX5^{low} cells was sufficient to permit IL-12-driven tumor suppression (**Fig. 16a**). Even minimal numbers of sorted NKp46^{+}NK1.1^{low}DX5^{low} cells (1x10^{3}) could potently halt tumor growth (data not shown). As expected, the same population isolated from *Il12rb2*$^{-/-}$ mice on the other hand had no impact on the tumor regardless whether the tumor did or did not release IL-12 (**Fig. 16a**). Due to the rarity of this cell type in the spleen (less than 1% of all splenocytes), 6x10^{3} co-injected unfractionated splenocytes, obtained from *Rag1*$^{-/-}$ mice failed to reintroduce tumor suppression in the *Il12rb2*$^{-/-}$ mouse (**Fig. 16b**). Flow cytometric analysis of the leukocyte pool from wt mice showed, that only ~3-5 % of total splenocytes are NKp46^{+} and within this NKp46^{+} population the majority is NK1.1/DX5^{high} (~80%), while only (~10-20%) belong to the double low/negative population (data not shown). Notably, sorted true cNK cells (NKp46^{+}NK1.1^{hi}DX5^{hi}) did not control tumor growth (**Fig. 16c**), which supported the idea that IL-12 acts through the recruitment of NKp46^{+} LTi cells and not cNK cells.

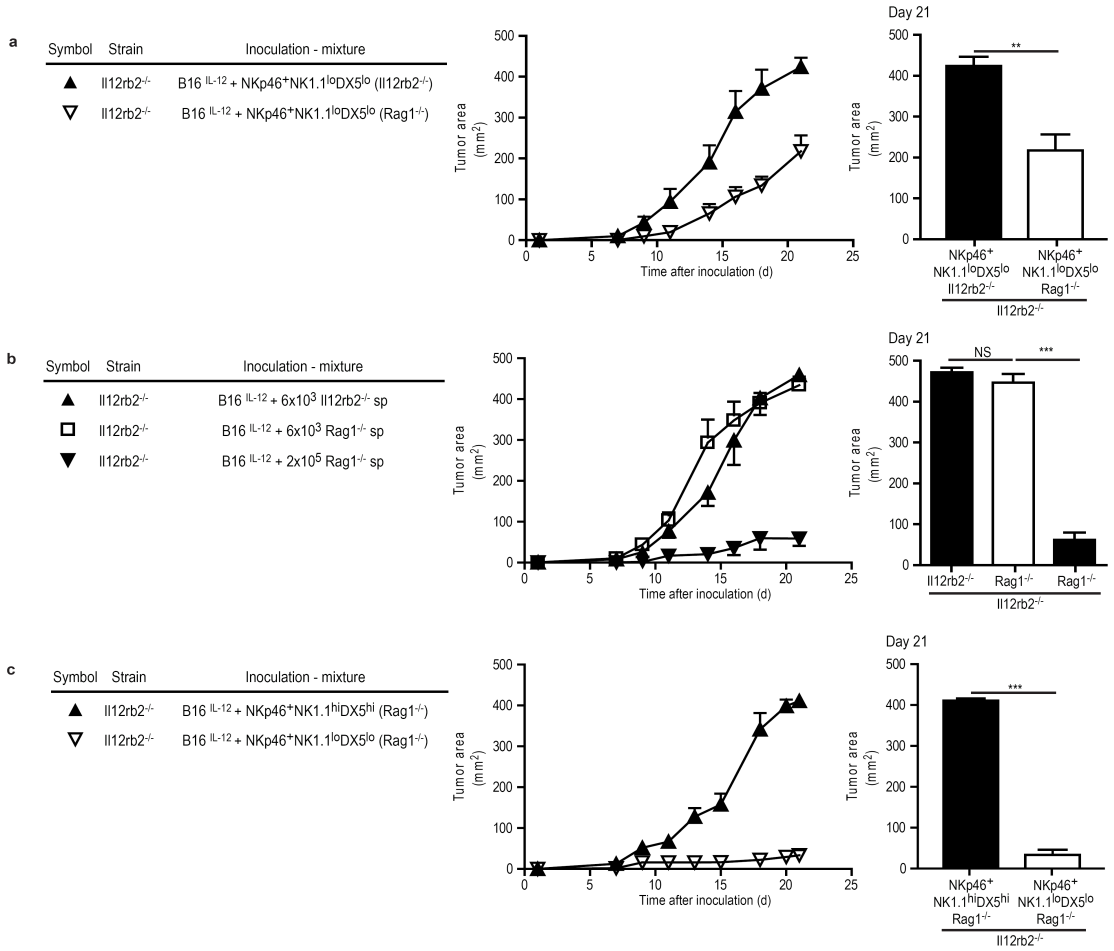

Figure 16 co-implantation experiments with NKp46$^+$ LTi cells and cNK cells

(**a**) Tumor suppression in *Il12rb2*$^{-/-}$ mice given subcutaneous co injection of 2×10^5 B16–IL-12 cells and 6×10^3 sorted NKp46$^+$NK1.1^{lo}DX5^{lo} cells obtained from *Rag1*$^{-/-}$ or *Il12rb2*$^{-/-}$ mice ($n \geq 6$ mice per group); cells were sorted by flow cytometry with a high-yield pre-sort for NKp46$^+$ cells followed by a low-pressure, high-purity sort for NKp46$^+$NK1.1^{lo}DX5^{lo} cells. Data are representative of at least two experiments. (**b**) Tumor suppression in *Il12rb2*$^{-/-}$ mice given subcutaneous co injection of 2×10^5 B16–IL-12 cells with 6×10^3 splenocytes obtained from *Rag1*$^{-/-}$ or *Il12rb2*$^{-/-}$ mice or with 2×10^5 splenocytes from *Rag1*$^{-/-}$ mice (n = 6 mice per group). (**c**) Tumor suppression in *Il12rb2*$^{-/-}$ mice given subcutaneous co injection of 2×10^5 B16–IL-12 cells and 6×10^3 sorted NKp46$^+$NK1.1^{hi}DX5^{hi} cNK cells or NKp46$^+$NK1.1^{lo}DX5^{lo} cells obtained from *Rag1*$^{-/-}$ mice ($n \geq 6$ mice per group); cells were sorted by flow cytometry with a high-yield pre-sort for NKp46$^+$ cells followed by a low-pressure, high-purity sort for NKp46$^+$NK1.1^{lo}DX5^{lo} cells or NKp46$^+$NK1.1^{hi}DX5^{hi} cells. Data are representative of two experiments. Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

RORγt-dependency

In contrast to cNK cells, NKp46$^+$ LTi cells develop independently of IL-15 but are dependent on the transcription factor RORγt [258] [257]. Even though NKp46$^+$ LTi cells share some characteristics with cNK cells, they represent a functionally and developmentally distinct lineage [270] [271].

NKp46$^+$ LTi cells require RORγt for their activity and development [239] [230]. We therefore took advantage of a *Rorc*-driven fate-mapping reporter mouse. *Rorc*-Cre × *Rosa26*-stop-eYFP, hereafter called '*Rorc*-eYFP mice', express the Cre recombinase in Rorγt$^+$ cells, leading to the excision of the stop-cassette and consequent expression of enhanced yellow fluorescent protein (eYFP) driven by the *Rosa26* promoter. Tumors from *Rorc*-eYFP mice inoculated with B16–IL-12 cells contained tumor-invading CD3$^-$eYFP$^+$NKp46$^+$ cells (**Fig. 17a**). We confirmed that result by immunofluorescent histology (**Fig. 17b**).

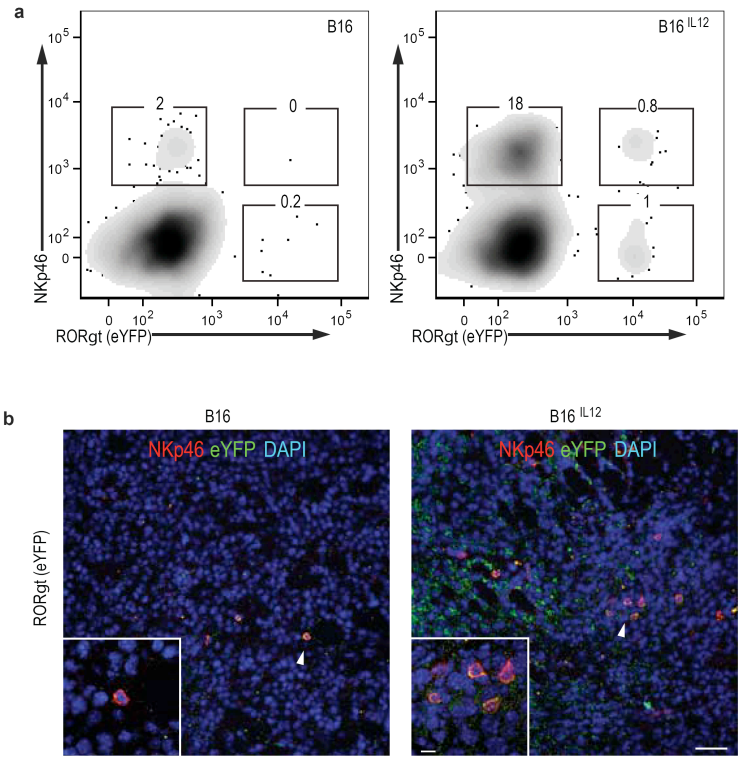

Figure 17 NKp46$^+$LTi cells invade the B16–IL-12 tumor

(**a**) Flow cytometry analysis of NKp46 and eYFP in tumor cells from *Rorc*-eYFP mice 3 weeks after inoculation with B16 cells (left) or B16–IL-12 cells (right), gated on live, singlet CD45$^+$ and CD3$^-$ cells. Numbers above outlined areas indicate percent NKp46$^+$eYFP$^-$ cells (top left), NKp46$^+$eYFP$^+$ cells (top right) or NKp46$^-$eYFP$^+$ cells (bottom right). (**b**) Cryosections from the tumor periphery of tumor-bearing *Rorc*-eYFP mice 3 weeks after inoculation with B16 cells (left) or B16–IL-12 cells (right), stained with anti-GFP-YFP (green) and anti-Nkp46 (red), detected with fluorescent secondary antibodies, and counterstained with the DNA-intercalating dye DAPI. Scale bars, overview pictures = 100 μm (main images) or 10 μm (insets).

Il12rb$^{-/-}$ mice co injected with B16–IL-12 cells and unfractionated *Rorc*$^{-/-}$ leukocytes failed to mediate tumor suppression (**Fig. 18a**). In contrast, co injection of sorted eYFP^{+}NKp46^{+} LTi cells with B16–IL-12 cells into IL-12R-deficient mice reintroduced IL-12-mediated tumor suppression to these mice (**Fig. 18b**). Together these findings demonstrate that LTi cells are necessary to repress subcutaneously tumor growth initiated by IL-12.

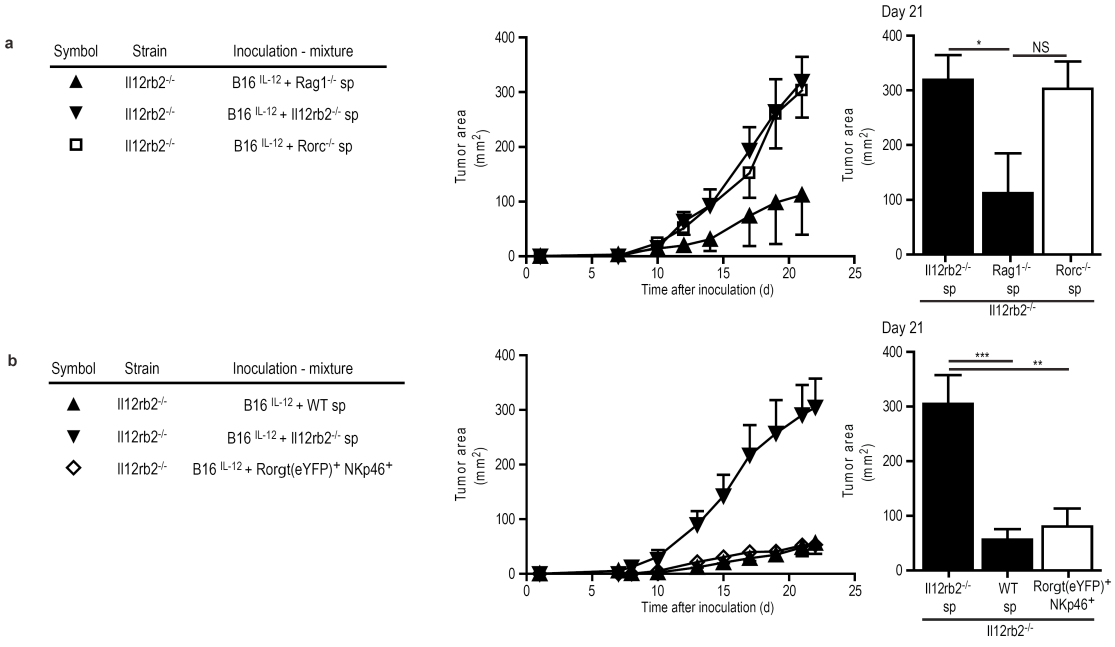

Figure 18 NKp46^{+} LTi cells suppress tumor growth in an IL-12R and RORγt dependent way.

(**a**) Tumor suppression in *Il12rb2*$^{-/-}$ mice given subcutaneous co injection of 2×10^{5} B16–IL-12 cells at a ratio of 1:1 with unfractionated splenocytes from *Rag1*$^{-/-}$, *Il12rb2*$^{-/-}$ or *Rorc*$^{-/-}$ mice ($n \geq 6$ mice per group). Data are representative of three experiments. (**b**) Tumor suppression in *Il12rb2*$^{-/-}$ mice given subcutaneous co injection of 2×10^{5} B16–IL-12 cells with 2×10^{5} splenocytes from wild-type mice, 2×10^{5} splenocytes from *Il12rb2*$^{-/-}$ mice or 6×10^{3} sorted NKp46^{+}RorceYFP^{+}CD3^{-} cells from *Rorc*-eYFP mice ($n \geq 4$ mice per group); cells were sorted with a high-yield pre-sort for eYFP^{+} cells followed by a low-pressure, high-purity sort for NKp46^{+}eYFP^{+}CD3^{-} cells. Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

IL-12 mediated Tumor Suppression is independent of IL-17, IL-22, IFNγ and Perforin

In vitro stimulation of LTi cells

To determine how NKp46^{+} LTi cells mediate tumor suppression, we enriched mice for LTi cells by injecting IL-7–anti-IL-7 complexes into Rorc-eYFP mice. It has been recently shown that the biological activity of IL-7 can be increased by combining IL-7 with an anti-IL-7 Ab [282]. We isolated LTi cells after 7 d, cultured them on a monolayer of OP9 mouse bone marrow stroma feeder cells and stimulated them for 24 h with recombinant IL-12. IL-12 induced the expression of IFN-γ and lymphotoxin-α but not of IL-22 or IL-17 (**Fig. 19**).

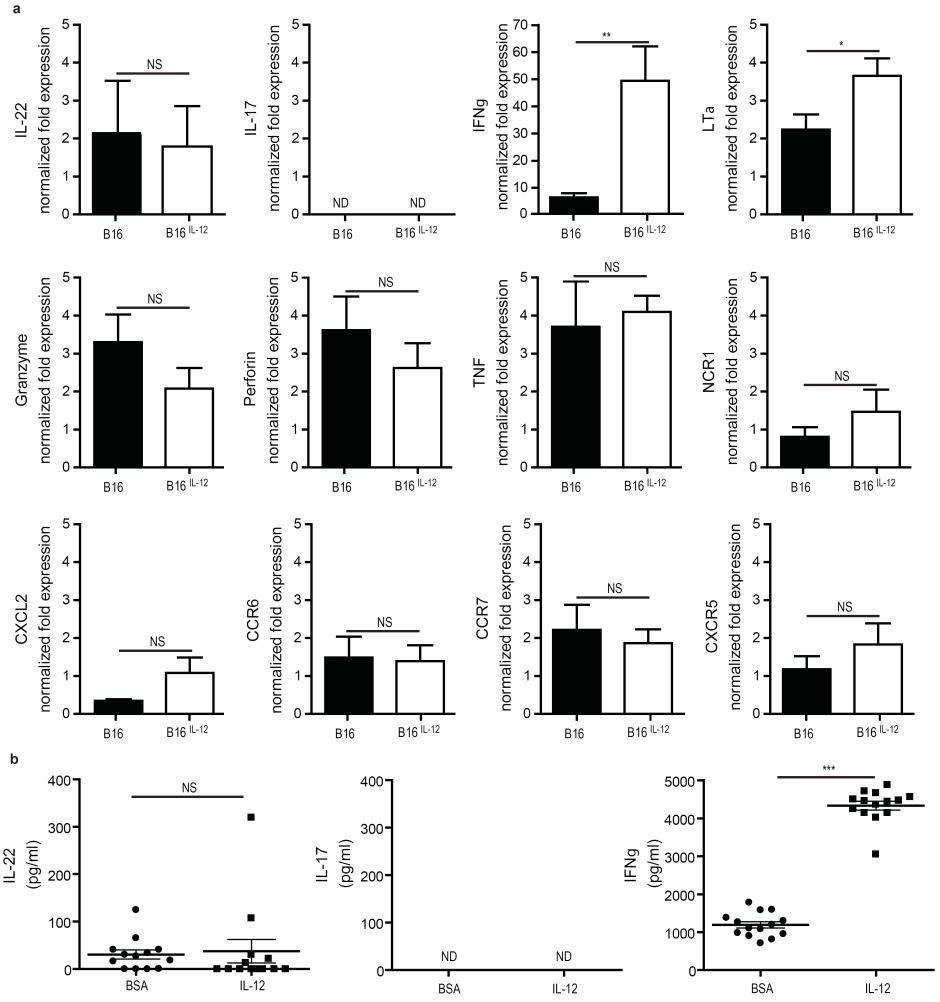

Figure 19 IL-12 induces IFNγ and LTá but not IL-22 or IL-17 by LTi cells

Rorc-EYFP mice were treated with á-IL-7/IL-7 complex as described by Schmutz *et al.* [283]. LTi cells were isolated using a high-yield pre-sort for EYFP^{+} followed by a low-pressure, high purity sort for EYFP^{+}CD3^{-}. The cells were cultured on a non-confluent monolayer of OP9-feeder cells in the presence of 10ng/ml of IL-7 with or without 2.5 ng/ml rIL-12 for 24h prior to (**a**) isolating RNA and qRT-PCR analysis. (**b**) Supernatants were collected and analyzed by ELISA for IL-22, IL-17 and IFNã. Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

IL-22 and IL-17

Even though it has been described that the NKp46^{+} LTi cell produce IL-22 as well as IL-17, the IL-12 mediated tumor suppression seems to be independent of both cytokines, since the B16-IL-12 tumor growth was still repressed in the *Il22*$^{-/-}$ (**Fig. 20a**) as well as in the *Il17*$^{-/-}$ (**Fig. 20b**) mouse. This findings tally with the in vitro data, where the cells did not produce IL-22 or IL-17 upon stimulation with IL-12.

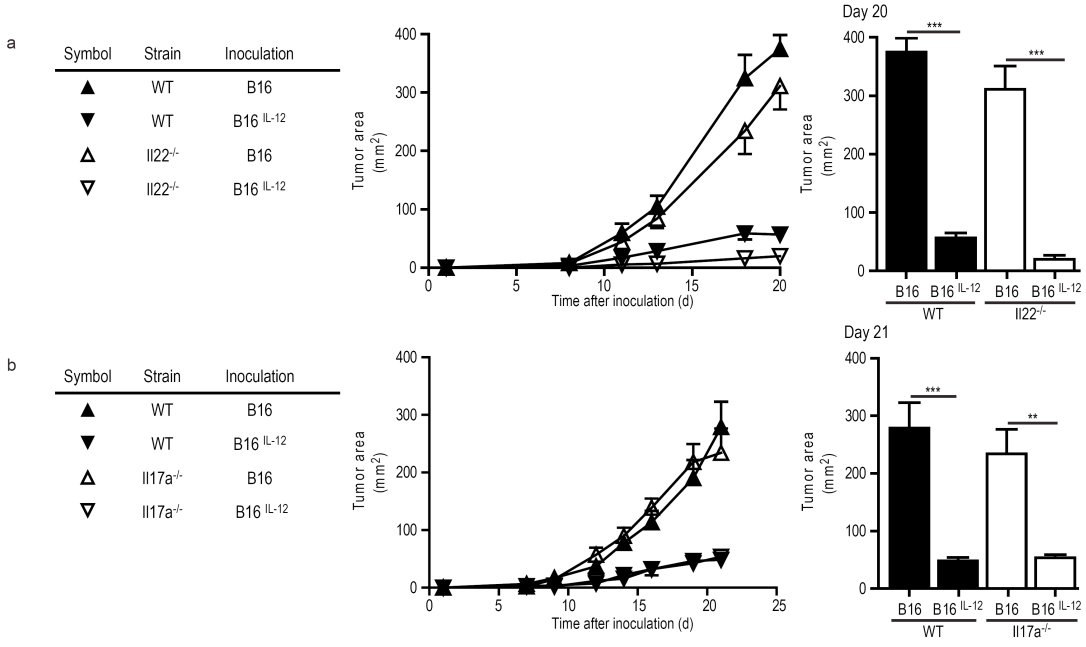

Figure 20 The role of IL-22 an IL-17 in IL-12-mediated tumor suppression

(**a**) and (**b**) Tumor suppression in wild-type mice (**a**,**b**), *Il22*$^{-/-}$ mice (**a**) or *Il17a*$^{-/-}$ mice (**b**) given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells (n = 6 mice per group). Data are representative of at two experiments. Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

IFNγ and perforin

More likely candidates for mediating direct tumor suppression and cytotoxicity are IFNγ and perforin. As shown previously IFNγ was clearly upregulated upon IL-12 stimulation of LTi cells *in vitro*. However, as in wild-type mice, B16-IL-12 tumors were successfully repressed in mice deficient in IFNγ (**Fig 21a**), IFNγ receptor (**Fig. 21b**) or perforin (**Fig. 21c**), which indicate that these mediators do not have a non-redundant function.

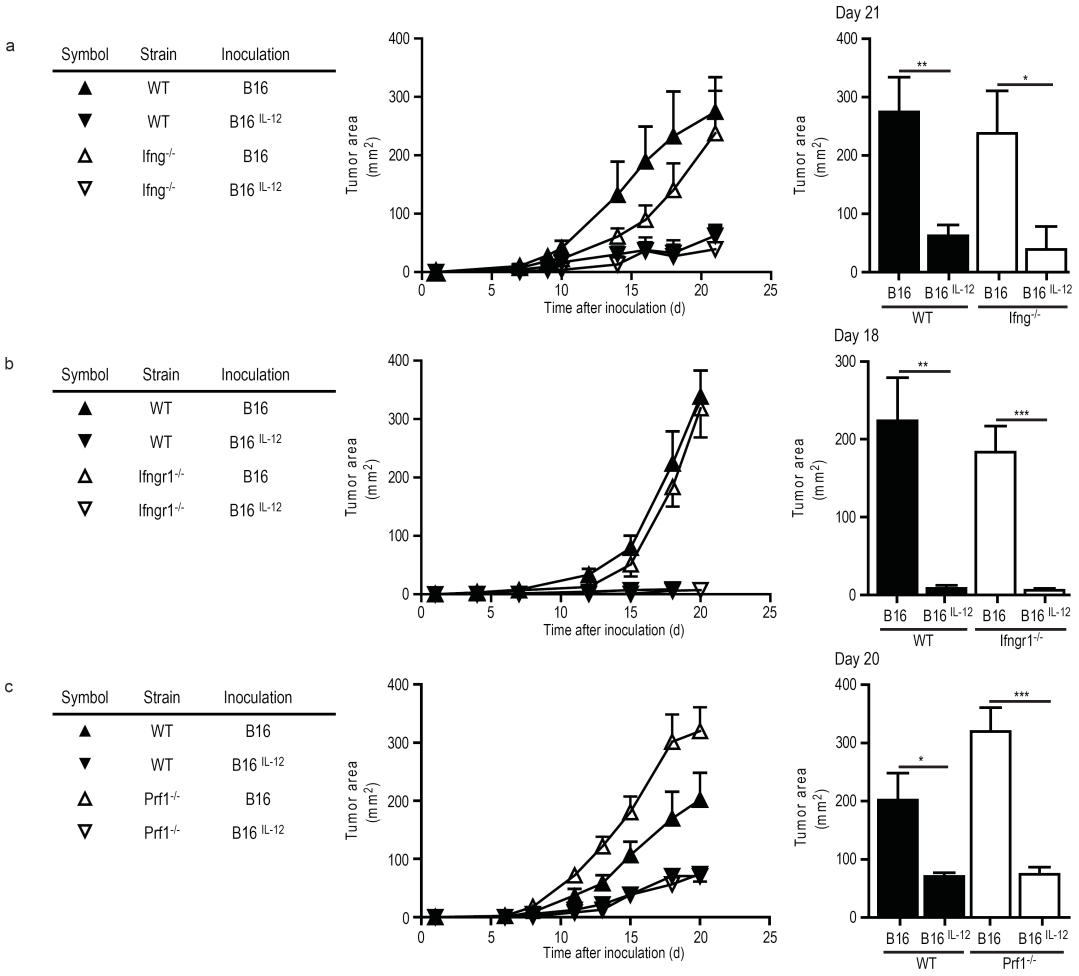

Figure 21 The role of IFNγ an Perforin in IL-12-mediated tumor suppression

(**a**) and (**b**) Tumor suppression in wild-type mice (**a**,**b**), IFN-γ-deficient (*Ifng*$^{-/-}$) mice (**a**) or IFN-γ receptor–deficient (*Ifngr1*$^{-/-}$) mice (**b**) given subcutaneous injection of 2×10^5 B16 or B16–IL-12 (n = 5 mice per group). Data are representative of at least two experiments. (**c**) Tumor suppression in wild-type and perforin-deficient (*Prf1*$^{-/-}$) mice given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells (n = 6 mice per group). Shown are the mean and s.e.m.; NS, not significant. *P < 0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

NKp46+ LTi Cells up regulate Adhesion Molecules in Tumor Vasculature

Adhesion Molecules

Angiogenesis is a characteristic feature of cancer, but the vasculature generated by tumors differs considerably from regular blood vessels [284]. One prominent feature of tumor vessels is their relative paucity of adhesion molecules such as VCAM and ICAM [285] [286], which conveys the apparent tumor immune privilege [287]. There is precedence for the ability of LTi cells to initiate the expression of adhesion molecules in stromal cells during lymphogenesis [239], and we thus investigated the ability of IL-12-driven NKp46$^+$ LTi cells to alter the expression of VCAM and ICAM in tumor vessels. In growing B16 tumors, VCAM was undetectable and ICAM staining is weak on CD31$^+$ vessels. In contrast, B16–IL-12 tumors showed slightly more ICAM immunoreactivity (**Fig. 22**) and much higher in VCAM expression (**Fig. 23**). As expected, in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, both B16 and B16–IL-12 tumors were vascularised (CD31$^+$) but lacked VCAM expression and had only minimal expression of ICAM, which demonstrated that expression of adhesion molecules requires NKp46$^+$ LTi cells.

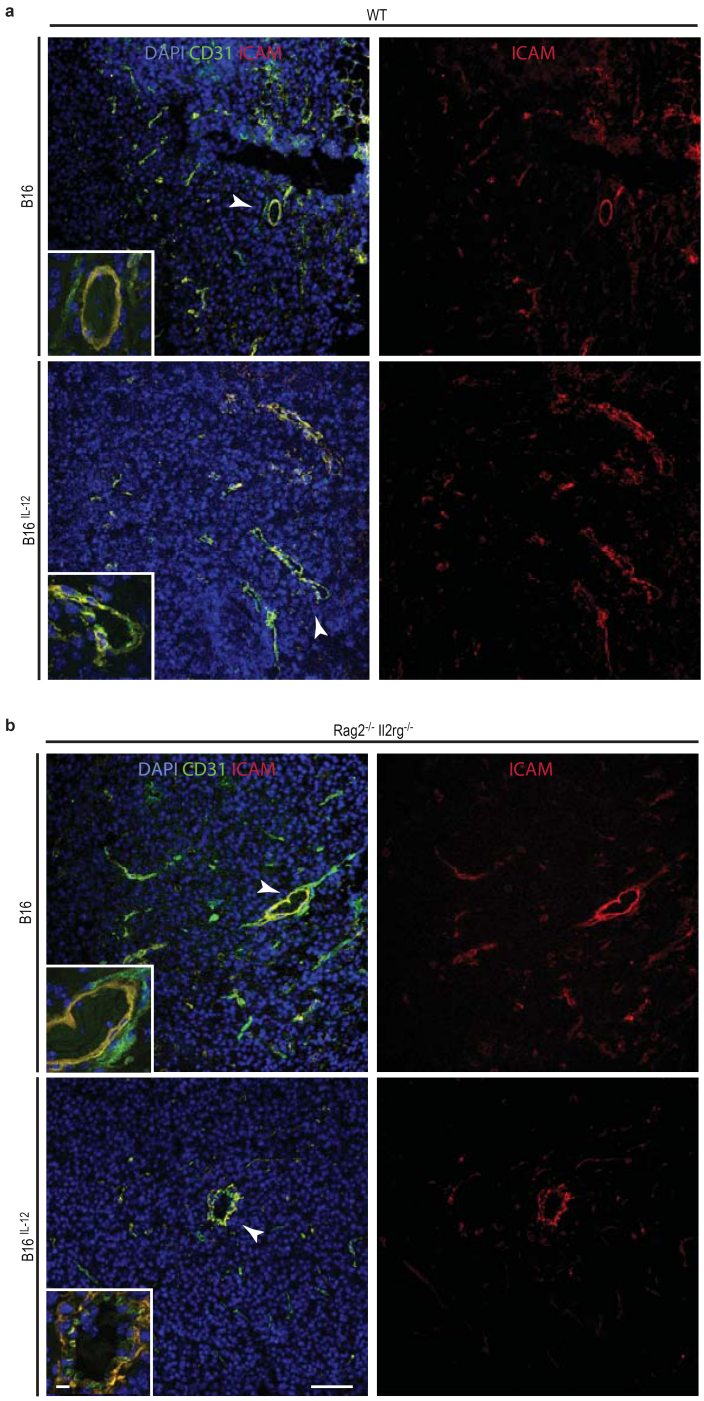

Figure 22 LTi cells alter the tumor microvasculature (ICAM)

(**a)** and (**b**) Microscopy of tumors from wild-type (**a**) or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (**b**) mice, injected with B16 or B16–IL-12 cells, stained for CD31 (green), ICAM (red) and counterstained with DAPI (blue). Left (merged images), overview of tumor margins; arrowheads indicate regions enlarged in inset (bottom left). Right, red single-channel images. Scale bars, 50 μm (main images) or 10 μm (insets). Data are representative of at least two experiments.

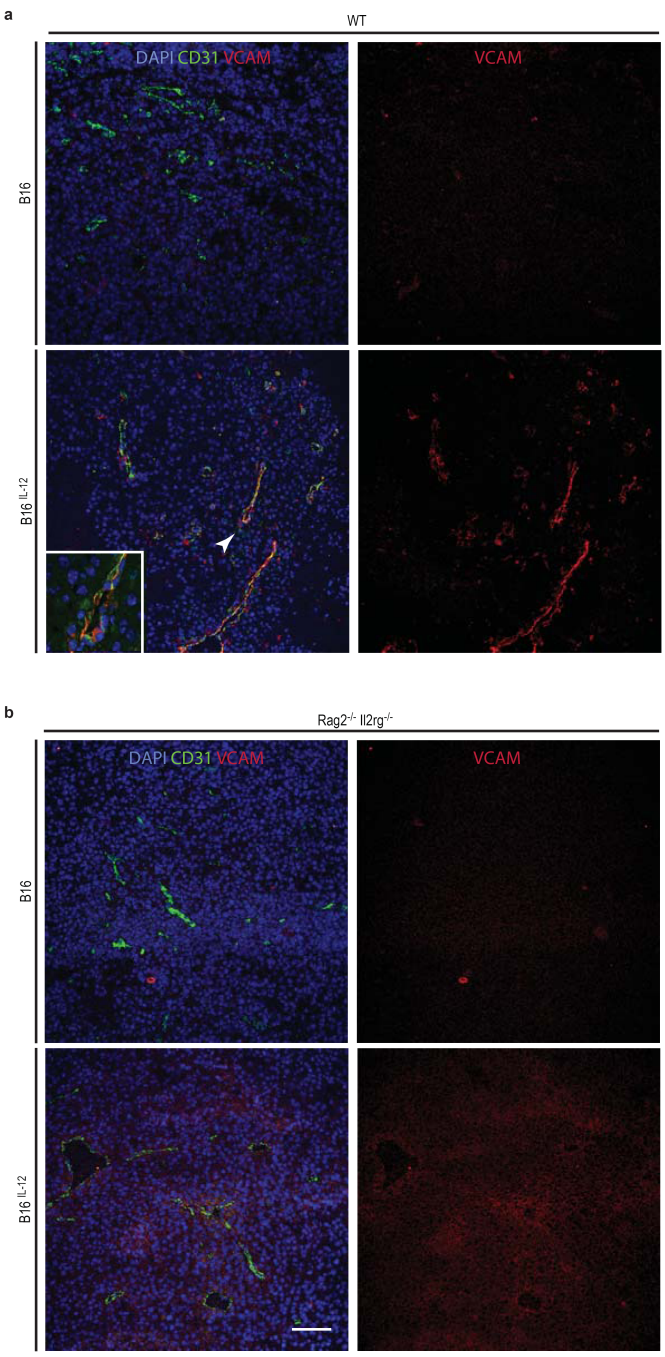

Figure 23 LTi cells alter the tumor microvasculature (VCAM)

(**a)** and (**b**) Microscopy of tumors from wild-type (**a**) or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (**b**) mice, injected with B16 or B16–IL-12 cells, stained for CD31 (green), VCAM (red) and counterstained with DAPI (blue). Left (merged images), overview of tumor margins; arrowheads indicate regions enlarged in inset (bottom left). Right, red single-channel images. Scale bars, 50 μm (main images) or 10 μm (insets). Data are representative of at least two experiments.

Lymphotoxin

During lymphogenesis, signaling through the lymphotoxin-β receptor is involved in up regulating vascular adhesion molecules [239]. Furthermore, therapy with fusion proteins of antibody and lymphotoxin-α can eradicate established pulmonary metastases and subcutaneous tumors [288]. However IL-12 retained the ability to suppress tumor growth in *Ltbr*$^{-/-}$ mice (**Fig. 24**), which suggested that lymphotoxin-β receptor signaling is not absolutely required for IL-12-induced tumor suppression. Together our data indicate that IL-12-stimulated LTi cells alter the tumor microvasculature, leading to up regulation of adhesion molecules and subsequent tumor control.

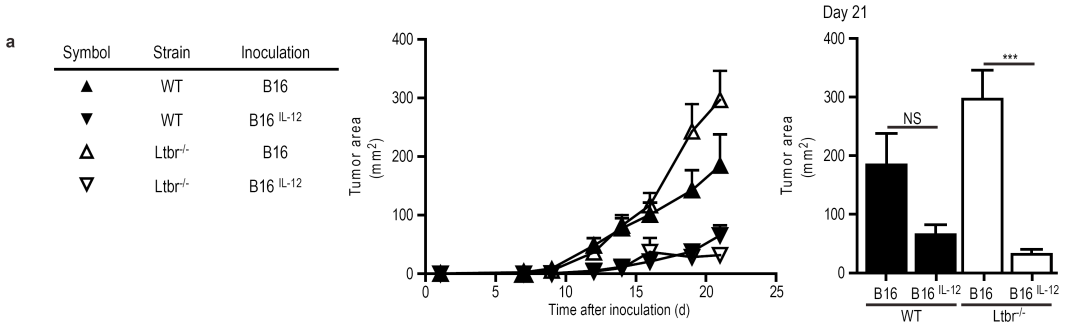

Figure 24 The role of LT's in IL-12-mediated tumor suppression

(**a**) Tumor suppression in wild-type and lymphotoxin-ß receptor–deficient (*Ltbr*$^{-/-}$) mice given subcutaneous injection of 2×10^5 B16 or B16–IL-12 cells ($n \geq 3$ mice per group). Shown are the mean and s.e.m.; NS, not significant. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (Student's *t*-test).

DISCUSSION

Over the past 20 years, IL-12 has been shown to have powerful tumor-suppressing effects in a variety of animal models, yet the mechanistic underpinnings of this phenomenon have never been fully understood. Indeed, there are several known mechanism by which the immune system may fight cancer in combination with IL-12. Production of IL-12 at the tumor site may induce the rejection of neoplastic cells by $CD4^+$ and $CD8^+$ T cells, NK cells or NKT cells. But even in the same mouse model of malignant melanoma (transplantation of B16 melanoma cells into C57BL/6 mice), different mechanism of both the innate and adaptive immune responses have been linked to the mediation of IL-12-induced tumor suppression [179] [177] [31] [189]. Further, a published study has shown that IL-12 fails to repress tumor growth in mice lacking the common γ-chain of the IL-2 receptor [189]. It was concluded that a rare population of tumor-killing DCs is unable to develop in the absence of the common γ-chain and thus that this cell type is responsible for the IL-12-dependent elimination of melanomas in mice [189]. But here we have shown that neither the adaptive immune response nor NKT or cNK cells were involved in IL-12-induced retardation of subcutaneous tumor growth. And also macrophages and DCs failed to mediate tumor suppression *in vivo* in our reconstitution experiments in which we implanted the tumor cells together with IL-12-responsive leukocytes.

By precise examination of the tumor mass through flow cytometry and histology we clearly detected an increase in immune cells infiltrating the tumor when releasing IL-12. And experiments with bone marrow chimeras of wt and *Il12rb2*$^{-/-}$ mice supported the notion of the tumor killing activity initiated by the activation of an immune cell through IL-12. However, tumor inoculation experiments with *Rag1*$^{-/-}$ mice clearly showed that even in the absence of T, NKT and B cells IL-12 is able to initiate the suppressing effect. In addition *Rag1*$^{-/-}$ mice were depleted of cNK cells by the application of anti-NK1.1. or anti-GM1. Both experimental setups showed no loss of the IL-12 – mediated effect, suggesting no involvement of NK cells. To finally proof the cNK cell-independent effect of IL-12, experiments with *Il15ra*$^{-/-}$ mice were performed. The development of cNK cell is dependent on IL-15 and *Il15ra*$^{-/-}$ mice therefore lack NK cells. The experiment clearly showed again no difference between *Il15ra*$^{-/-}$ and wild-type mice when injected with melanoma cells secreting IL-12. *Il15ra*$^{-/-}$ mice though have been shown to contain relatively few residual cNK cells, which proliferate after infection with cytomegalovirus. Now, one could argue that this small population of leftover cNK cells is activated by IL-12. However, an equally small cNK population is also present in *Il2rg*$^{-/-}$ mice [289]. The finding that IL-12-driven tumor suppression functioned in *Il15ra*$^{-/-}$ mice but not in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice showed that this residual cNK cell population did not contribute to tumor suppression here. Accordingly, IFNγ did not seem to have a non-redundant function within the IL-12-mediated tumor suppressor effect. Mice deficient in IFNγ or its receptor showed no significant variation in the tumor growth curve compared to

wild-type mice when inoculated with melanoma cells secreting IL-12. Nor did perforin seem to play a crucial role in the repression of the tumor growth, since mice deficient in perforin again had comparable suppression as wild-type mice.

As already mentioned, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$mice have been shown to lack almost all cNK cells. However, in contrast to *Il15ra*$^{-/-}$ mice, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$mice additionally lack secondary lymphoid tissue as well as LTi cells. Recently, an additional LTi-like cell type has been described on the basis of NKp46 expression, as well as IL-22 expression, in mucosal sites [257 259 258]. The function of this cell type in immunity, however, remains mostly elusive. In fact, the role and function of LTi cells, apart from forming the anlagen for lymph nodes during development, is a subject of debate [255].
Here we have demonstrated that IL-12 injected into or secreted by the tumor mass was able to recruit/expand this NKp46^{+} LTi subset leading to efficient tumor suppression. Apparently, LTi cell activity in tumors alone is sufficient to initiate an inflammatory cascade leading to the suppression of tumor growth. With reconstitution experiments into *Il12rb2*$^{-/-}$ mice with sorted NKp46^{+} LTi, we were able to re-introduce the abolished IL-12-mediated anti-tumor effect in these mice. Even minimal numbers such as 6000 implanted cells were capable to render the experimental phenotype. The same small number of unfractionated leukocytes failed to control tumor growth, which tally the finding that only minimal numbers of LTi cells can be found in adult organs. This again demonstrated that NKp46^{+} LTi cells are the most potent and essential population of IL-12-responsive leukocytes capable of tumor suppression *in vivo*.

It is now widely believed that LTi cells are the precursors of this newly identified population because of their dependence on RORγt [257 259 258]. Tallying with this believe, we were not able to re-introduce the IL-12-mediated effect in *Il12rb2*$^{-/-}$ mice with implanted leukocytes from *Rorc*$^{-/-}$ deficient animals. Even though it has been shown that NKp46^{+} LTi cells produced IL-22 and IL-17, the IL-12-mediated tumor suppression seemed to be independent of these effector cytokines. Nor did the isolated and *in vitro* IL-12-stimulated LTi cells produce IL-22 or IL-17, neither showed the *Il22*$^{-/-}$ and *Il17*$^{-/-}$ mice an impaired IL-12-mediated tumor suppression. In contrast, IL-12 stimulation of LTi cells in vitro led to higher expression of IFN-γ, which has been widely linked to tumor control. But as already mentioned, we failed to identify a non-redundant function for IFN-γ and its receptor. It is likely that one single effector molecule is not responsible for the tumor-suppressive function of LTi cells but instead that IL-12 elicits the transcription of a specific set of factors that ultimately lead to tumor control and/or eradication.

Here we postulate that adult LTi cells, which are present in secondary lymphoid tissues, can be recruited to form crude lymphoid structures in an IL-12-induced fashion. It has been suggested, that in the lymphoid-organ development clustering of LTi cells and VCAM1^{+} stromal cell play a central role. Triggering of the lymphotoxin-ß receptor (LTßR) signaling through the interaction of LTi cells and stromal cells then leads to the production of chemokines involved in lymphoid organogenesis and the further up regulation of VCAM1 expression. The result of this is the accumulation of more hematopoietic cells [239]. Tumor vasculature usually possesses no adhesion molecules and therefore

prevents the infiltration of immune cells to a minimum. Clearly, we observed a strong up regulation of VCAM1 on tumor vessels in the B16–IL-12 tumor compared to control tumors together with an increased infiltration of immune cells. However, regarding the responsible LTßR signaling for the upregulation of VCAM in the anlagen of a lymph node, the blockade of LTßR signaling by the use of *Ltbr*$^{-/-}$ mice did not lead to any defect in the IL-12-mediated tumor suppression.

The mechanism by which NKp46^{+} LTi cells alter the tumor microenvironment remains to be fully resolved. A published study has shown a correlation between expression of the chemokine CCL21 by tumors and recruitment of LTi cells [290]. A causal relation between the presence of LTi cells and functional tumor suppression, however, was not established [290]. Although it is unclear at present whether NKp46 expression is crucial for the tumor-suppressive function of LTi cells, we propose that IL-12 converts a subpopulation of LTi cells in the tumor into NKp46^{+} LTi cells, which in turn changes the tumor microenvironment from anti-inflammatory to pro-inflammatory. That idea is supported by the finding that the presence of NKp46^{+} LTi cells correlated well with the up regulation of adhesion molecules in the tumor microvasculature. The precise mechanism by which this is achieved requires further investigation. Notably, cNK cells had a non-redundant role in controlling the homing of melanoma cells into the lungs of mice injected intravenously with B16–IL-12 cells. The abundance of cNK cells in the lungs relative to that in the skin could explain this phenomenon. Alternatively, the relative paucity of melanocytes in the lungs could account for the greater immunogenicity of lung-invading melanoma cells than that in the skin, in which the melanoma precursor cells are common. Unexpectedly, however, IL-12-mediated rejection of glioblastomas from the central nervous system relies on T lymphocytes, and neither NK cells nor LTi cells are essential (data not shown).

Systemic administration of IL-12 in a clinical trial for kidney cancer has been halted because of severe adverse effects [291]. Although we confirmed that systemic delivery of recombinant IL-12 was a potent tumor suppressor, we found that local administration into the tumor was equally effective. Depending on the tissue, the presence of IL-12 in the tumor alone seemed to be sufficient for the recruitment of NKp46^{+} LTi cells, which in turn efficiently repressed tumor growth. Local *IL12* gene therapy of malignant melanoma in patients has also yielded promising results in established tumors [292]. The recognition that IL-12 activates a local immune response in the primary melanoma via NKp46^{+} LTi cells sheds new light on the therapeutic potential and mechanism of action of IL-12 in cancer. IL-12-induced alteration of tumor vessels may also lead to the subsequent recruitment of T lymphocytes or B lymphocytes to the tumor site and trigger the exposure and recognition of tumor antigens. This possibility is now being explored, with plans to combine IL-12-mediated local tumor suppression with blockade of co inhibitory molecules such as CTLA-4 on T cells for targeting the formation metastases [287] [293]. More broadly speaking, these findings may guide the development of combination tumor therapies to make tumors more accessible for targeted immune therapy and vaccination to establish long-term antitumor immunity in patients.

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